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PRODUCTION OF DISEASE-FREE SEED
Theodore P. Dykstra

CHEMICAL TESTS IN THE DIAGNOSIS OF PLANT VIRUS DISEASES
R. C. Lindner

TECHNIQUES FOR THE OBSERVATION AND ISOLATION OF SOIL MICROORGANISMS
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H. T. Clifford

FUTURE CONTENTS ON INSIDE BACK COVER

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PRODUCTION OF DISEASE-FREE SEED

THEODORE P. DYKSTRA[1]

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INTRODUCTION

The production of disease-free seed involves several factors. One major problem is to produce seed free from seed-borne infection from viruses, fungi and bacteria. Another problem concerns conditions which can adversely affect germination and undermine the seed to such an extent that plants of reduced vigor result. For example, the common pea mosaic virus, although not seed-transmitted, may cause flower sterility or a stimulation of pod setting in lupine (161). Such pods contain seed of inferior quality and of half the normal size.

The factors favoring disease development in relation to the production of disease-free seed are discussed in sections entitled (*a*) Seed-borne Organisms, (*b*) Disease Resistance and Variation in Fungi, (*c*) Environment, (*d*) Field Sanitation, (*e*) Storage, (*f*) Plant Quarantine, and (*g*) Seed Certification. In some cases illustrations which, in

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themselves, may not have any significant bearing on the development of disease-free seed are given, but they are used to present examples of fundamental biological principles which can operate in producing disease-free seed.

In the work of producing disease-free seed, more knowledge is needed of the extent to which diseases are seed-transmitted. A better understanding is also needed of the behavior of pathogens affecting dormant seed during storage and of the conditions favoring the occurrence of a disease in crops raised from infected seed.

One critical period in plant growth, from a disease standpoint, is from seed germination to seedling stage and young-plant growth. Physical and chemical seed treatments reduce loss from disease at this time by destroying pathogens borne on seed surface and below the seed coat and soil-borne pathogens near seed in seedbeds. After a stand of a crop plant has been established, it is frequently necessary to provide continuing protection to plants with sprays and dusts during the growing period. Studies on conventional seed treatments and on spraying and dusting are not included in this article, since these subjects have been reviewed in previous papers (41, 167, 173).

Although potatoes are increased vegetatively, the seed pieces are considered in the trade more in the sense of seed than of propagation stock, and potatoes are, therefore, discussed in this paper.

SEED-BORNE ORGANISMS

Disease-causing organisms infecting seed are viruses, fungi and bacteria. Preventing transmission of these organisms is important in producing disease-free seed.

Viruses

Viruses may be transmitted in the embryo of the seed but are not transmitted externally on the seed coat as the seed coat has no direct connection with the developing seedling (108). Only through mechanical means during germination could a virus in the seed coats infect the seedlings (33, 34).

Crowley (33) demonstrated that seed transmission of common bean mosaic virus depends on the ability of the virus to infect the microspores or embryo sac before fertilization. It is now generally accepted that there are no plasmodesmatal connections to embryos, and the likely

path for movement of virus between cells is by way of the plasmodesmata (32).

Of several hundred plant virus diseases so far described, Crowley (33) found only 45 that are seed-borne. In only four species does seed transmission normally exceed 50 percent. Although a strain of tobacco mosaic virus from pungent pepper (*Capsicum frutescens*) is seed-transmitted, the embryo is not at first infected, but becomes so by contamination from the infected testa during germination.

Tobacco mosaic in affected tobacco plants is distributed through the placental structures and even reaches the ovules (2). However, mosaic in tobacco does not occur as a result of embryonic transmission directly from the mother plant during seed development. Some barrier guards against embryonic infection or continuation of the disease from parent to seedling as germinable seeds from plants affected with the disease have always produced normal, healthy plants.

In curly top of sugar-beet and tobacco (10), the virus is most abundant in the vascular region of the seed but cannot be recovered from the embryo. A seedling developing from an infected seed can utilize the stored food without acquiring any of the virus.

Crowley (34) inoculated Early Red Valentine Kidney and Pinto beans *(Phaseolus vulgaris)* and soybean with bean southern mosaic virus, Lincoln tobacco and three varieties of soybeans with tobacco ring spot virus, and Compana barley and Selkirk wheat with barley-stripe virus. The viruses were all able to infect embryos and gametes only during the early stages of their development. The results support the view that the absence of seed transmission of some highly infectious viruses may be due to their inability to infect and survive in the young gametophytic and meristematic tissues.

The virus of southern bean mosaic is present in all flower and fruit parts of systemically infected bean varieties (103). Both seed coat coat and embryo originally contain the virus. As the seed matures, dehydration occurs, and the virus in the embryo is inhibited (18). In seed transmission tests, the seedlings derived from mature seed did not contain the virus with the exception of Logan, State and Brittle Wax varieties. These three varieties showed five, two and three percent southern mosaic, respectively. Seedlings from immature seed showed a high percentage of infection. The average percentage of infection from these was 50, whereas for Logan it was 80.

The virus causing elm mosaic was found in pollen from an infected

tree (15). Tests were conducted to make all possible pollination combinations between healthy and infected parents. The resultant seedlings established the fact that infections may be transmitted from pollen to seed.

One hundred per cent seed transmission of tobacco ring spot virus, the cause of the bud blight disease in soybean, has been observed. The efficiency of seed transmission depends on the time of infection of the seed-bearing plants. Seventy-eight percent of the plants showing symptoms at 38 days gave seed transmission, and 91 percent of the seed of these plants was infected as compared with 54 percent infected seed on 15 percent of the plants that develop symptoms at 46 days. Between 46 and 64 days of age the plants showing transmission averaged 13 percent, and transmission by these plants averaged 10 percent (7). During this period there was a tendency toward decrease in percentage of plants showing transmission and percentage transmission.

Tobacco ring spot virus and the tomato ring spot virus induce similar symptoms in inoculated soybean of the Lincoln variety. The ring spot virus on soybean was known to be seed-transmissible (38). In a seed transmission test, tobacco ring spot was transmitted to 82 percent of the seedlings, and the tomato ring spot to 76 percent of the seedlings (67). These results were cited as the first report of seed transmission of the tomato ring spot. However, Jones (64) had already reported that tomato ring spot in *Cineraria* is seed-transmissible.

The virus causing yellows of beets was found to be seed-transmissible only occasionally (22, 129), and the rate of seed transmission may not exceed 0.1 percent. The virus of a disease resembling virus yellows was transmitted from a particular cross selected from good commercial stock. Of the 143 plants grown from seed, 47.5 percent showed the disease. It is presumed that the yellows virus was present in at least one parent and that it penetrated the seed of the progeny.

In a test to determine the incidence of red-node virus in bean seed obtained from currently infected plants, very little seed-borne infection was noted (156). Average infection with the red-node virus in Pinto beans was 1.4 percent. Maximum infection was six percent in one sample of University of Idaho #72 beans. Seed from infected plants grown from infected seed produced an increased amount, average 26.5 percent, of the disease. No uniformity in occurrence of the virus within seeds in individual pods or among different pods on infected plants could be observed.

From 1.1 to 11.7 percent of seeds in four lots transmitted the virus causing peach ring spot. Seed transmission of peach necrotic leaf spot virus from 3.8 to 9 percent of the seeds in three lots was found. The evidence indicated that all seedlings infected with peach necrotic leaf spot virus also were carrying peach ring spot virus (166).

The principal virus disease of lupines in southeastern United States is caused by a strain of bean yellows mosaic virus (29). This disease seems to be similar to the one described as sore-shin, a virus disease of blue lupines in New Zealand. Some of the five virus diseases described by Klesser (75) in South Africa would appear to be caused by strains of the bean yellows mosaic virus. The extensive leguminous host range of bean yellows mosaic makes this virus a potential threat to many crops grown in southeastern United States. Some strains of this virus have been isolated from nonleguminous hosts (50). The fact that bean yellows mosaic is transmitted through 6.2 percent of the seed of diseased yellow lupine plants indicates that a sufficient source of the inoculum is available to cause an entire field to be almost uniformly infected by harvest time. Mastenbroek (87) reported five percent seed transmission of a virus causing the mosaic disease of yellow lupine in the Netherlands. This virus may have been bean yellows mosaic virus or a closely related strain.

There is definite evidence of difference in degree of seed transmission. A decreasing percentage of seed transmission occurs as plants are infected closer to the time of flowering. The group infected through the seed and the group infected when the plants were four weeks old were the highest in transmitting the virus. The plants infected after flowering failed to produce any mosaic diseased seedlings. No evidence has been obtained of seed transmission of the dandelion mosaic virus to lettuce (69). On the other hand, a yellow mosaic of lettuce (164) plants grown at New Delhi, India, was transmitted readily from seed of infected plants because 30 percent of the plants raised under controlled conditions developed the disease.

The Harosoy soybean variety begins to flower when about 56 days old, with the flowering period averaging 34 days. Therefore, the relation shown between the amount of seed transmission and the time of symptom expression is probably a relation between seed transmission and flowering, or fertilization. Nelson (104) found that seed from plants infected after flowering rarely transmit the virus of bean mosaic.

Tests conducted in the Netherlands (160) demonstrated that lettuce

mosaic virus in *Lactuca seriola* from a number of European sources was readily transmitted through the seed, but the level of transmission varied from 0.2 to 50 percent.

Common mosaic of lettuce is seed-transmitted (31, 52, 69, 101). While seed transmission occurs to the extent of about three percent, considerable variation exists among varieties with respect to this characteristic. The variety Bibb may have eight percent or more infected seed, whereas varieties Chestnut and Early Giant may be entirely free from seed-borne virus. The percentage of seed transmission varies from 0.21 to 14.18 percent among individual plants of the same variety.

The principal distinction between the common bean-mosaic virus and the yellow bean mosaic virus is that the common bean mosaic virus is seed-transmitted (58) and the yellow bean mosaic virus is not. A previously unreported strain of common bean-mosaic infecting Great Northern U1-123, immune from bean virus 1 (*Marmor phaseoli* Holmes) and from the variant strain reported from New York and Idaho, was found to be seed-borne (36). Symptoms caused by the virus strain were not sufficiently different from those incited by the type strain of virus 1. Bean varieties carrying dominant resistance to the type strain of bean virus 1 were either resistant or susceptible to the newly found strain.

Transmission of a muskmelon mosaic virus through melon seed has been demonstrated (120, 163). A high percentage of seed transmission occurred in progeny from diseased plants. The virus was readily transmitted experimentally (120) through seeds of *Cucurbita moschata, Cucumis flexuosus, C. melo* and *C. pepo* but not through cucumber (*C. sativus*).

Although Kreitlow and Hunt (77) were unable to demonstrate definitely that seed transmission of alfalfa mosaic virus or bean yellows mosaic virus to Ladino white clover, a substantial reduction in flowering and seed production occurs among plants infected with these viruses. The average percentage reduction in yield from virus infection was 42.

McKinney (90) and Eslick (45) reported that the barley stripe-mosaic is caused by a seed-borne virus. This is a potentially serious disease of wheat and barley. Since information concerning the virus in winter wheat is meager, investigations were conducted to determine extent of damage to winter wheat varieties. Although spring observations of the disease showed reduced stands, reduced vigor, and pro-

nounced foliar symptoms, the virus transfer from diseased to disease-free winter wheat was infrequent. Under such conditions the economic importance of the disease was considered negligible (49).

An extensive host study of the tobacco ring spot virus, employing both host reaction and serological tests, revealed that tobacco ring spot virus is yielded by *Ambrosia artemisifolia, Daucus carota, Erigeron strigosus, Rumex acetosella, Taraxacum officinale* and *Trifolium repens,* which are symptomless carriers of the virus (159). Although infected dandelion (*Taraxacum officinale*) plants show no virus symptoms, subsequent tests have shown that an infected dandelion plant transmits tobacco ring spot virus through its seed. Dandelion may therefore be added to tobacco, soybean, and petunia as plants that transmit this virus through seed.

During an eight-year period, thousands of seedlings of lime, grapefruit, citron, and sweet lime were examined for symptoms of tristeza virus infection. There has been no record of transmission of the virus through the seed although the parent trees were definitely infected (88).

Seed analysts have reported necrotic spots on cotyledons of lettuce seedlings and considered these indicative of inferior quality. Studies to determine the nature of this malady revealed that cotyledonary spotting of lettuce seed is a manifestation of physiological drought (47). The tissues of developing lettuce embryos become desiccated as a result of moisture stress if maximum daily temperatures in excess of 90° F. occur over an extended period and moisture is low.

Since potatoes are propagated asexually, the principles underlying disease transmission differ from those pertaining to transmission through seed (42). Morphologically the potato tuber is an underground stem, and the organisms causing systemic diseases are present in the above-ground parts as well as in the underground stems. During the dormancy period, after potatoes have been harvested and placed in storage, the organism may remain quiescent and resume activity again when the tuber or cut seed is planted.

As far as known, all potato viruses except purple top wilt are transmitted by the tubers. If infection of a plant by a virus takes place late in the growing season, only part of the tubers may be infected because the virus is unable to penetrate all parts of the plant before it matures. For the same reason, occasionally the stem end of a tuber is infected and the bud end is healthy. Generally there are no reliable direct indica-

tions of virus diseases in the tubers. With few exceptions their presence can be determined only by an examination of the growing plants. The main exceptions are early infections by the spindle tuber and witches'-broom viruses which result in the production of small or abnormally shaped tubers. Net necrosis is a symptom of witches'-broom and also of current-season infection with leafroll virus in some varieties. Plants affected with yellow dwarf often produce small, misshapen tubers, which, in cross section, show small, necrotic areas scattered throughout the flesh.

Purple top wilt (79) is caused by a virus transmitted by the six-spotted leafhopper (*Macrostleles divisus*) from asters and other hosts of aster yellows. The disease symptoms are very pronounced. An internal necrosis of tubers extending from the stem end is characteristic of the disease, but may be absent. Occasionally tubers from infected plants are flabby. The virus does not survive in the tubers, but tuber progenies of plants affected with the disease are less vigorous than those of healthy plants.

Alfalfa mosaic virus occurs naturally in crop plants such as alfalfa, beans, celery, peas, pepper and potato. It produces a great variety of symptoms in potato ranging from calico to severe stem and tuber necrosis (54, 111, 112). Although not transmitted in the true seed of its host plants, it is transmitted in potato tubers.

Fungi

Seed-borne diseases are often common on wheat, oats and barley. Pathogens on these crops include species of *Helminthosporium: H. sativum* on wheat and barley; *H. avenae* and *victoriae* on oats; and *H. teres* on barley (55, 150). Bacteria found on wheat are *Bacterium herbicola aureum* and *Pseudomonas* sp. (61).

Podosporiella verticillata is a fungus which causes a rare seed-borne disease of wheat (168). It was occasionally isolated from samples of common and durum wheats and was found to be synonymous with *Helminthosporium cyclops*. Seed naturally infected does not germinate. When wheat and oat inflorescences are sprayed with a spore suspension, the seed developing later, although outwardly healthy, does not germinate or produces only stunted seedlings.

Brown foot rot of wheat *(Calonectria nivalis)*, an uncommon seed-borne disease in Britain, was found for the first time on barley in Scot-

land (107). It was the principal seed-borne disease of this crop. *Septoria nodorum,* which is frequently seed-borne on wheat, causes a brown foot rot at the base of the seedlings. Tests with treated seed indicated that leaf spot of oats and brown foot rot of all three seedlings are often transmitted in treated seed even when mercurial fungicides are applied.

It was not until 1957 that Reed (126) verified seed transmission of the causal organism of scald of barley, *Rhynchosporium secalis.* This is a major foliage disease of barley in many regions. Caldwell (16) had reported negative results in attempts to demonstrate overwintering of scald on seed and the dissemination of the organism by infested seed. Dillon (39) also concluded that the fungus is not seed-borne. Skoropad (136) collected seed from threshed barley taken at random from fields where scald had appeared on the leaves. These showed an average disease incidence of two percent.

The head blight of small grains caused by the scab fungus *Fusarium graminearum* was excessive and widespread in New York in 1957 and 1958. The cool humid weather favored development of this and other species of seed-inhabiting fungi. Similar conditions prevailed in some out-of-State areas from which seed stocks are procured for local planting.

The blind seed disease of ryegrass has been studied extensively (48, 171). Seed of perennial ryegrass *(Lolium perenne)* infected by *Phialea temulenta* germinates very poorly. The histology of infected grain shows that whenever the scutellum becomes infected, the seed usually fails to germinate. The heavily seed is opaque, whereas slightly infected seed is generally translucent.

To determine whether it will be worthwhile to harvest crops that are practically worthless because of low germination, samples of a crop should be tested two to three weeks after flowering and again when the seed is mature. Reports on the immature samples give the proportion of seeds infected up to the time of examination and permit recommendations on subsequent sampling. In reports on mature seed, an estimate of the germinating capacity is given.

Flax is susceptible to several seed-borne diseases (85, 100, 102, 150). A species of *Macrosporium* is carried as mycelium on the seed coat. Upon germination of infected seed, the fungus infects the cotyledons and spreads to the hypocotyls. Since there is a relation between the degree of infection and the incidence of disease in the crop, a study

was initiated by using seeds infected with *Polyspora lini* and with *Colletotrichum linicola*. From seeds of several varieties there was a range of contamination with *P. lini* from 0 to 35 percent and with *C. linicola* from 0 to 46 percent. The crops from seed infected with *P. lini* did not develop serious disease if seed contamination was less than five percent. Stormut cirrus seed with eight to 35 percent seed contamination showed maximum infection at all stages of growth. Maximum attacks occurred when seed infection was 13 percent in Linal Dominion, 32 in Linal Monarch, and 21 in Linal Crown. A survey of flax seed produced in the United Kingdom and covering 5000 samples showed that these were infected with *Colletotrichum linicola, Polyspora lini, Botrytis* sp., *Phoma* sp. and *Fusarium lini*. The flax seed produced in the south and east of England was freer from seed-borne parasites than that from the wetter northern and western parts of the British Isles. *C. linicola* proved to be the most dangerous seed-borne pathogen.

A disease survey showed that flax seed from east and southeast England was relatively free from *Colletotrichum linicola, Polyspora lini* and *Phoma* sp., but infection was greater in the western and northern districts of the Pennines. *Botrytis cinerea* infection was usually the highest in coastal and upland areas.

Tests of seed from other countries indicated the possible importance of *Polyspora lini* in Denmark, of *B. cinerea* and *C. linicola* in the Netherlands, and of *C. linicola* as a contaminant of oil-flax seed in Sweden.

There are at least 32 parasitic diseases of soybeans (150), and 11 of a reported 13 seed-borne diseases of this host occur in Canada. Harvested soybeans have been found to be infected with many fungi (72, 73, 99, 121, 153). The fungi and their relative prevalence on the flowers, pods, and seeds varied with season, with location within the plants, and with varieties. *Alternaria* spp. were isolated most frequently from the pistil and stamens of unopened and opened flowers and from style and stigma remains adhering to pod tips. *Cercospora kikuchii* and *Phomopsis sojae* comprised the highest percentage of isolates from seed within pods. From 7.3 percent of 3800 unopened and opened flowers plated out over a three-year period, fungus isolates were obtained. From 68.4 percent of 5200 styles and stigmas from pod tips plated out, fungus isolates were obtained. Of 11,800 seeds from within pods plated out over a four- to six-year period, fungus isolates were obtained from 44.1 percent.

It has been assumed that purple stain of soybean is caused by *Cercospora kikuchii*. However, Jones (63) found that 10 of 13 species of *Cercospora* incited typical purple stain of soybean seeds, and the seed coat harbors the fungus.

Anthracnose of blue lupine, caused by *Glomerella cingulata,* has been prevalent in the Southern States (37). Under controlled conditions this disease reduced seed germination to 40 percent. Thirty percent developed into normal plants and the remaining ten percent showed typical anthracnose lesions on the leaves and stems.

A considerable number of pathogens were associated with forage-legume seed produced in Oregon (78). Even though seed-producing areas in Oregon are theoretically ideally suited for the production of "disease-free" seed because of low summer precipitation and low relative humidities, transmission of pathogens by seeds does occur. This was illustrated strikingly by the number of organisms repeatedly detected in seed lots. These organisms were: *Mycosphaerella pinodes* and *Botrytis cinerea* on field peas; *Phoma herbarum* var. *medicaginis, Fusarium roseum* and *Stemphylium botryosum* on alfalfa; *B. cinerea, F. roseum, Phoma trifolii* and *S. botryosum* on alsike clover; *B. cinerea, F. roseum, P. trifolii, Sclerotinia sclerotium* and *S. botryosum* on crimson clover; and *Ascochyta pisi* on hairy vetch. The levels of infestation of seed lots by pathogens were generally rather low.

The production of alfalfa seed in Minnesota has declined during the past 20 years to negligible quantities (30, 62, 71) owing to the prevalence of *Ascochyta imperfecta,* the cause of black stem. Severe symptoms of black stem develop on the stems and leaves of the inoculated plants in seven days; the flowers, buds, peduncles and pods become infected. Dark lesions develop on the receptacles and pedicels of young pods within five days. Thirty days after pollination and infection, the pods are flat, dry, and straw-colored. Seeds from infected plants are uniformly light in weight, brown, shriveled, and non-viable. The disease can virtually destroy a seed crop of alfalfa if sufficiently heavy infection occurs at the flowering stage. The quality of the remaining seed is greatly impaired through reducing viability and carrying the organism.

One thousand samples of vegetable seed from Great Britain and the United States were examined (115). The seed was so heavily infected with parasitic fungi that 50 species of fungi, including several new species, were identified. These fungi rendered some seed unfit for sowing and the remainder required treatment.

From 47 samples of agricultural seed received in Ottawa, seven species of *Alternaria* were isolated, six of which were pathogenic (150). *A. tenuis,* which was isolated from all the seeds tested, appears to have a world-wide distribution. *A. brassicae* was isolated from seeds of cabbage, cauliflower, rape, and radish of Canadian, United States and Dutch origin. *A. dauci,* the cause of a serious leaf blight of carrots, was on only two samples of seed from Nova Scotia and Ontario. *A. raphani* was isolated from radish seeds originating in Ontario, Quebec and British Columbia, and those imported from California, New Jersey, Ohio and Pennsylvania. *A. radicina* was isolated frequently from carrot seed and occasionally from parsely. *A. oleracea,* which is of wide geographical distribution, was isolated from seeds of swedes, broccoli, cauliflower, cabbage, radish and runner beans. Seed treatment has consistently failed to control blackstem because the organism infects the living embryo.

During routine seed testing in Denmark in 1953-58 mycelium was detected in 23 of 1,045 lots of *Brassica* seed, including cabbage, cauliflower, kale and swede (103). It is suggested that dead flower parts may be an important source of seed infection.

Phoma lingam is a serious disease of swede and turnip in New Zealand (83). A survey in 1957 and 1958 showed 223 of 263 seed samples to be free from infection. In the diseased samples there was generally only 0.1 percent infection. Approximately 50 percent of the infected seed did not germinate. The fungus remained viable for 14 months in some stored seed but died after eight months in some.

Although eggplant seeds have been assumed to be carriers of *Verticillium* wilt fungus (66) and isolation of this fungus from seed of this and other crops has been reported (152), its economic importance has been questioned. Burton and deZeeuw (14) found it extremely difficult to induce infection of seeds in fruits growing on plants infected with the wilt fungus. Only one of the infected fruits produced seeds that carried *Verticillium.* Since this fruit was cracked and partially decomposed, contamination from the soil was a distinct possibility. The results of numerous laboratory greenhouse and field experiments over a three-year period require the conclusion that seed transmission of *Verticillium* wilt in eggplant is highly improbable.

Ring spot of crucifers, generally restricted to coastal and other moist regions of the world, was found on summer oil rape *(Brassica campestris)* in an inland area with an annual precipitation of 15 inches. The

causal fungus, *Mycosphaerella brassicola,* was isolated from diseased seeds (162). This inland evidence supports the seed-borne nature of the organism.

Colletotrichum piperatum, causing anthracnose of pepper, has resulted in excessive losses in pepper fields in Delaware (139). *C. piperatum* and *C. capsici* were found capable of surviving in or on dry pepper seed for at least nine months.

Although Cardona-Alverez and Walker (17) concluded that the causal fungus of angular leaf spot of bean is not carried in or on the seed, further studies (110) have caused this decision to be reversed. Seed from infected plants showed reddish-brown discoloration of the coat. About 50 percent of the discolored seed developed fungus growth identified as *Isariopsis griseola.* Infected seed when planted in sterilized soil developed typical symptoms on the cotyledonary leaves after 13 days. The controls from disease-free seed remained healthy.

A severe outbreak of angular leafspot of sesame has been observed in Panama (46). The disease completely burned the leaves. This disease was found to be seed-transmitted and most likely introduced into Panama on seeds from Nicaragua.

Although fenugreek is a very old cultivated plant in both the Old and New World, the *Cercospora* disease did not appear in older cultures of this plant. Newest findings, particularly from analyses of seed samples from various countries, indicate that *Cercospora traversiana* must be a native in Asia Minor and India and was only lately introduced into western countries. From seed samples from different countries it appears that some new and particularly vital races or strains of *Cercospora traversiana* have started their spread from the Near East and are now ready to invade North America (81). *Cercospora traversiana* is a typical seed-borne fungus, forming mycelium inside the host plant. It begins to develop during germination of the seed and produces conidia abundantly on the seed coat. Infected seeds are not killed immediately but give the fungus shelter for overwintering and for far-distant distribution.

Brown rot fungi (*Phytophthora* spp.) are present in the testas of seeds from infected citrus fruits and will infect the nursery seed beds and be spread to nursery rows in transplanting. Balled trees from such nurseries constitute an important source of infection to clean land (76).

In potatoes, fungus diseases such as powdery scab *(Spongospora subterranea),* black scurfs *(Rhizoctonia solani* and *Synchytrium endobioti-*

cum) late blight (*Phytophthora infestans*) and wilts (*Fusarium oxysporum, F. eumartii, F. solani, F. avenaceum, Verticillium albo-atrum*) can all be transmitted by tubers, but development of disease is greatly influenced by environment.

Bacteria

Thaung et al. (154) found that, contrary to general opinion, bacterial blight of lima bean is commonly present in the United States west of the Continental Divide and the causal organism is present on seed from these areas.

Symptoms in general agreement with those described for bacterial leaf spot of squash were found to be seed-transmitted (91). Infections are generally restricted to 10 to 15 percent of the seedlings from one lot of seed.

Three bacterial diseases affect potatoes and are usually carried over in the seed tubers (42). Blackleg, caused by *Erwinia phytophthora,* is responsible for heavy losses in some potato-growing sections. In many cases tubers developing from infected plants may produce tubers that do not show any rot, but these healthy-looking tubers may carry the disease over to the next season. It was shown in a test in Oregon that 60 percent of the plants developed blackleg when apparently sound tubers from hills infected with blackleg were planted. If weather conditions are unfavorable for the disease, infected tubers will develop into healthy plants. Brown rot, or bacterial wilt, *Xanthomonas solanacearum,* is also transmitted by seed tubers, but its occurrence is restricted to the South Atlantic and Gulf Coast States. Ring rot *(Corynebacterium sepedonicum)* is an extremely infectious disease and almost world-wide in distribution. The causal organism is carried over in potato tubers and gives rise to diseased plants.

DISEASE RESISTANCE AND VARIATION IN FUNGI

Breeding for resistance is the most important phase of an improvement program of crops plants. Resistant plants do not transmit disease through seed. Resistance also is the most effective way of avoiding secondary infection in plants grown from disease-free seed. From a practical point of view, planting disease-free seed in areas where a specific disease occurs in epidemic form is of doubtful value, since plants will become infected regardless of whether disease-free or infected seed is

planted. Completely resistant varieties fail to contract disease even when exposed to infection.

The essential character of true resistance lies in the protoplasm of a plant and is independent of inoculation accidents. However, the different degrees of disease resistance displayed by plants must not be regarded as fixed and absolute. Virulence of the pathogen, the age and condition of the plant, and the environment with its many effects on both host and pathogen, all must be suitable for maximum susceptibility to be expressed. Disease resistance needs to be defined, therefore, not only in terms of organism-host relationship but also in terms that include the environmental conditions as they influence both host and pathogenic parasite.

In testing for resistance countless seed have been grown and progenies selected in the field on the basis of cultural characteristics. After screening to obtain economic types, the remaining seedlings have to be exposed to severe infection by the pathogen. By means of the host plant, its varieties and segregates, it has been possible to demonstrate a wide range of variability in fungi. The complexity of the causal organism parallels that of the crop plant. We are thus concerned with the genetic make-up of the host as well as that of the parasite. Pathogens, especially fungi, germinate, grow, multiply, mutate, and hybridize, and, as a consequence, countless new types of fungi are being developed. It is necessary, therefore, to learn as much as possible about the variability of fungi. The problem of disease is often international (141) because plant varietes resistant to pathogens in one country often are completely susceptible to those in another.

Most of the original information on the variability of fungi has been obtained from the Plant Pathology Laboratory of the University of Minnesota where extensive investigations have been conducted on the behavior and variability of *Puccinia graminis,* which causes stem rust of small grains and grasses, and on *Ustilago zeae,* the causal organism of the common corn smut (8, 82, 109, 140, 142-146). The fundamental principles underlying the behavior of these pathogenic fungi, as revealed by these investigations, have been found by others to apply also to many other plant parasitic fungi (12, 25, 40, 59, 96, 105, 106, 124, 125, 127, 131, 149, 165). Since most species of plant pathogenic fungi are just as complex as crop plants, it is necessary to find out species differences. There is variation within the species of fungi that are alike in some characters and different in others, including the crop

plants they can attack. Within the fungi there are races that differ in their ability to attack certain varieties of a crop. The races in turn can be subdivided into ultimate subdivisions called "biotypes." A biotype is a genetically identical population of individuals. The test of genetic purity of a population of a pathogenic fungus is the consistency of its behavior. Many pathogenic fungi mutate on artificial media. Then, there is evidence that mutation occurs also in host plants in nature but may not be observed unless the mutation causes conspicuously different infection types or differences in color. It is more difficult to observe mutation for pathogenicity than for cultural characters.

The economic results obtained in developing varieties of sugar beets resistant to diseases have been spectacular (26-28). The sugar-beet industry has suffered near-disaster from certain diseases. The important diseases of sugar beet are curly top, a virus disease in the West and Southwest; leaf spot *(Cercospora beticola)*, a defoliation disease; and black root, a seedling disease-complex in which *Aphanocmyces cochlioides* is the most important pathogen. However, these diseases have been greatly reduced by an extensive disease-resistance program.

Probably no plant disease can produce such early and complete destruction as curly top of sugar beets. Within a few weeks, affected plants may become so diseased that farmers plow up the fields and abandon sugar-beet growing. In western United States, in curly-top epidemic years, as many as 5000 acres surrounding a factory have been abandoned. To meet the depressed situation of the western sugar-beet industry, curly-top resistant varieties were developed by making mass selections of the sugar beets surviving in the fields that were severely injured by curly top. Mass selections of this type were pooled to produce the first curly-top resistant variety, U. S. 1. Although only moderately resistant, U. S. 1 was outstanding in production in comparison with the almost complete failure of sugar-beet crops grown from European seed. U. S. 1 was quickly followed by other varieties. Each of these varieties was more resistant than the stock from which it was selected. New improved varieties were bred and quickly released to farmers.

When the seed of the curly-top resistant variety U. S. 1 was first released to beet-sugar companies, the requirement was that the reproduction of seed to supply growers be made in the United States subject to control by the Division of Sugar Plant Investigations. Whereas, formerly all sugar-beet seed was imported from Europe, now all the sugar-

beet seed required for planting the commercial sugar crop in the United States is produced here.

Mass selection, so successful with curly top, did not work with *Cercospora* leaf spot. Outstanding plants were not found in heavily blighted fields. Inbred strains, selected from European varieties of sugar beet, showed that a few strains were outstanding because of their milder reaction to the disease. From this experience it was evident that, to obtain leaf spot-resistant varieties, selection of strains showing resistance and continued inbreeding would be required until factors for resistance were stabilized within a strain. Breeding for leaf spot resistance resolved itself into producing as many distinct leaf spot-resistant inbreds as possible. Then, by a series of matings, those pairs that would give a strong heterosis response as well as leaf spot resistance were selected. By continued breeding, new leaf spot-resistant varieties have been introduced. One of these leaf-spot-free varieties equals in sugar production that of European varieties previously grown. Under conditions of leaf-spot exposure it is significantly superior to susceptible European types. In additional work, factors for resistance to downy mildew have been combined with factors for resistance to curly top; and resistance to leaf spot and to black root have been combined.

About a century ago a new and ravaging blight of potatoes became so serious that complete crop failure of this important food crop resulted in a famine in a number of European countries. Since then many plant scientists have devoted their efforts to the study of this disease, since it is recognized as a world-wide problem in the production of this basic food crop. During this century of study and struggle with *Phytophthora infestans,* attention has been devoted to finding or developing resistant potato varieties. In 1870 Darwin attempted, through the use of species hybrids, to produce varieties resistant to *Phytophthora* but was evidently not successful. The first American breeder to attempt control of late blight by the production of blight-resistant varieties was Chauncey Goodrich of Utica, New York. His work was based on a small quantity of South American potatoes received in 1851. Except in the work of Goodrich and a few others, resistance to late blight was not emphasized until 1910. A number of seedlings of the cross Chippewa × Katahdin, both of which are susceptible to blight, showed resistance. One of these seedlings displayed a fairly high degree of resistance but became infected during severe blight years. In addition

to partial resistance, it had also many desirable horticultural characters. It was introduced and named "Sebago" (148). It soon became evident that complete resistance could not be developed by using *Solanum tuberosum* only in making crosses.

It became necessary to take into consideration also the variability of the pathogen in the breeding of resistant potato varieties. At present it is known that a number of races of *P. infestans* exist in different parts of the world, that they are characterized by variability of pathogenicity or specialization, and that this variability is governed by gene mutations or other mechanisms within the organism. Some of the tuber-bearing species of *Solanum,* also known as wild potatoes and occurring in Mexico, Central America and Andean region of South America, were found to be resistant to some of the virulent races of *Phytophthora infestans.* An intensive program of hybridization was begun in both Europe and the United States with these collections (59, 96, 105, 106, 124, 125, 127, 149). Only one of these, *Solanum demissum,* could be used extensively. Hybridization of the other resistant wild species with commercial varieties often resulted in pollen sterility, incompatability, flower drop, and inheritance of the characteristics of the wild species. Some of these difficulties preventing hybridization were overcome in specific cases. The incompatibility between *S. demissum* with 72 somatic chromosomes and some varieties of *S. tuberosum* with 48 chromosomes could be overcome by the use of a bridging process. Thus the bridge species of *S. rybinii (S. phureja)* with a somatic number of 24 was crossed with *S. demissum* to produce an F_1 hybrid with 48 somatic chromosomes. The plant then was crossed with *S. tuberosum.* The resulting triple hybrid was fertile and could be used in additional back crossings to improve commercial characteristics.

Several species, including some clones of *S. demissum,* show a high resistance to *P. infestans* even though foliage lesions are produced on some of these species. These resistant-type lesions are slow spreading and often turn necrotic, and sporulation is sparse. Petiole and stem lesions tend to be superficial and do not kill the distal portion of the affected leaf or stem. Relatively few lesions develop on a plant, and, when they do, they are often on the lower, older leaves. The reaction demonstrated by resistant-type lesions is called "partial resistance." A high level of "partial resistance" is due to minor genes which condition the response of a plant to the races to which it is susceptible.

A variety such as Sebago may have a combination of minor genes that condition a slight degree of resistance to all races, even though it is susceptible to all races, including the common field race "O." A somewhat higher level of partial resistance is demonstrated by Alpha, Gineke and Voran, Dutch varieties. In the field they show a degree of resistance that is exhibited toward all races of the pathogen. The practical value of this type of resistance lies in the fact that these varieties have to be sprayed less often and less thoroughly for adequate protection against blight.

A second kind of resistance which might be called "racial resistance," is due solely to the presence of one or more of the major genes for resistance. When these genes are present, singly or in combination, they confer complete resistance to certain specific races. In areas where late blight occurs only occasionally, this type of resistance may be effective for many years. In locations where late blight infection is an annual occurrence, racial resistance is not sufficient. The history of many "resistant" potato varieties in other countries shows that once the varieties become fairly well distributed, they begin to blight. The ease with which the virulence of the pathogen can be altered makes it unlikely that complete immunity can be maintained in a given variety of potatoes over a long period. Breeding for partial or field resistance seems to be the answer to the late blight problem unless new factors for resistance can be found.

Resistance to common scab, as well as to blight, has been found in several wild species (123). *S. commersonii, S. chacoense, S. caldasii* var. *glabrescens* and *S. jamesii* were grown in soil heavily infested with the common scab organism, and for two successive years these *Solanum* species gave immune reactions.

Since the introduction of potatoes into Europe, species hybrids have been made so that *Solanum tuberosum,* as we know it, is heterogenous if it can be called a species. Its heterogeneity is shown by many characters among which are resistance to mild mosaic, rugose mosaic, latent mosaic, leaf roll, net necrosis, yellow dwarf, late blight of vines and tubers, common scab, wart, *Verticillium* wilt, ring rot, brown rot and blackleg. Seedling varieties and a few commercial varieties of potatoes have been developed that have resistance to one or more different diseases.

Varying degrees of resistance to plant disease have been developed by

breeding in most cultivated crop plants. The contribution of a disease-resistant variety generally includes also improvement in yield and quality.

ENVIRONMENT

The influence of environment and climatic conditions on development of plant diseases cannot be overestimated. A thorough comprehension of the effects of environment on the pathogen, the host plant, and the disease is basic to an understanding of the entire problem of disease in plants, its avoidance and control.

To perpetuate or to spread a disease, an organism must not only survive in time but must also be distributed in space. Fungi, as a rule, are not self-propelled. They depend, therefore, on chance distribution by wind, water movement, activity of birds and animals, insect carriers, and dissemination of host plants and plant parts. To a large extent the fungi are disseminated by spores, fragments of hyphae, and hard masses of mycelium, called sclerotia. The distance to which fungus spores may be carried by air currents is very great.

Some bacteria, spores of some fungi, and most viruses are adapted to dissemination by insects. The prevalence of a plant disease in such cases is often determined more by the influence of environment on the insect vector than by its influence on the disease itself. For example, bacterial wilt of cucurbits can be transmitted only by insects. Any weather condition that influences the abundance of the cucumber beetle must also influence the prevalence of the disease.

In spite of an abundance of the disease-causing organisms, infection occurs only when a plant is at a stage of development vulnerable to attack. The parasite that causes the disease must be in an infective stage. The environmental condition must also favor initial infection and development of disease.

Early attempts to determine scientifically under controlled conditions the effect of environment on development of disease was reported by Jones (65). These studies opened the way to detailed knowledge on effect of temperature on the parasite, the host plant, and the disease. For example, the *Fusarium* stage of *Gibberella saubenetii* is capable of parasitizing both wheat and corn seedlings. Corn is a high-temperature plant while wheat is a low-temperature one. Seedlings of these two host plants, when planted in soil infested by this parasite and exposed an an experimental range of temperature, showed significant

differences. At the low temperature the wheat seedlings remain healthy, while the corn seedlings become blighted. At high temperatures corn seedlings remain healthy, and the wheat seedlings become blighted. Since these investigations were dealing with but one parasite, the effect of temperature was on the host. Additional studies revealed that the embryonic parts of corn and wheat seedlings are sheathed by a protective covering through which invasion must proceed. The cell membranes of the sheath in the beginning are easily penetrated since they are composed chiefly of pectin. In normally balanced seedling-development, the cell membrane of this sheath passes quickly from the pectin stage to a condition of maturity in which celluloses, lignin, or suberin predominate and these wall elements resist invasion by the fungus. Any adverse condition which retards normal development must correspondingly retard this maturity process and prolong the state of relative susceptibility to fungus invasion. With wheat, low temperature is essential to normally balanced seedling development. High temperature disturbs this balance. Therefore, wheat is resistant at low temperature and blights at the high temperature. With the corn seedling, the normal temperature relations are reversed; therefore, the corn seedling is resistant at the high temperature and blights at the low temperature.

Resistance to powdery mildew *(Erysiphe graminis)* of susceptible barleys in the adult stage is a variable character that may be greatly affected by the environmental conditions under which the plants are grown in the pre-adult stage (151). When adult plants in the greenhouse were inoculated in the early heading stage in December and January, the cool, moist soil and air and subdued daylight in the greenhouse made the plants very tender and, hence, very susceptible. Adult plants became progressively resistant when inoculated in the heading stage in February, March and April.

Studies with other diseases have demonstrated conclusively that soil environment, such as temperature, moisture and composition, has a controlling influence upon all soil-borne parasites and that the factors comprising the environment determine the geographical distribution as well as the seasonal severity of these diseases (95). Onion smut serves as a striking illustration of the effect of a single environmental factor in the geographical distribution of a disease (65). After onion smut was first observed in New England, it soon became established in each intensive onion-growing center of the Northern States. Carloads of onion sets have been taken to the Gulf States where no smut

developed. Absence of onion smut in the South appears to be due to the fact that onion plants are susceptible to infection by the soil-borne spores of the smut fungus *(Urocystis cepulae)* for only a very short time in the seedling stage before emergence. Warm soils hasten the growth of the seedling and allow it to escape infection. In the South onion seed is planted in the fall and germinates in warm soil. Smut, therefore, is scarcely known in the South. By contrast the disease is important from Kentucky northward.

Temperature and moisture are the primary factors that limit epiphytotic development of northern anthracnose of red clover (23). The most favorable condition for disease development is a continuous period of cool, wet weather. The highest percentage of infection occurs in the narrow range of 18–22°C. Above the optimum temperature, infection is almost completely inhibited; below 18°, symptom development is greatly retarded. Among 4000 plants from shriveled and sound seed, when planted, 15 diseased seedlings occurred, indicating seed transmission.

Temperature greatly influences the development of damping-off and root rots of cotton seedlings (51). The disease incidence on young cotton seedlings at different temperatures is determined to a large extent by the relative growth rates of host and pathogen. Temperatures lower than optimum for the cotton plant favor the development of the organisms of damping-off and root rots of cotton seedlings. These organisms are *Colletotrichum* (*Glomerella*) *gossypii, Rhizoctonia solani, Thielaviopsis basicola* and *Aspergillus flavus.*

Seedling infection of barley by scald *(Rhynchosporium secalis)* is optimum at a soil temperature of 16°C., but decreases sharply at 20° and is completely absent at 22° (136). Dormant mycelium on scald lesions on the seed sporulates abundantly within 48 hours under cool, moist conditions. The coleoptile comes in contact with this inoculum as it grows under the diseased pericarp and lemma. The coleoptile develops a prominent scald lesion on its tip four to six days after emergence. At temperatures between 22° and 27° sporulation of *R. secalis* is retarded, but rapid growth of the plumule is favored. Under these conditions the seedling grows past the scald lesion before conidia are produced. Cooler temperatures retard plumule development, and sporulation of the fungus is favored. Thus inoculum is available to infect the coleoptile when it comes into contact with the scald lesion.

Wheat head blight has caused serious losses to wheat and other

cereals by lowering yield due to shriveling of the grain, or as a result of planting seed infected with *Helminthosporium sativum*. This causes impairment of germination and seedling emergence and reduces yield of the succeeding crop (4). Temperature greatly influences severity of this disease. Most favorable temperature conditions for infection and head blight development are 25°C. to 30°. Plants infected during the flowering stage develop equally severe head blight at 25° and 30°, but plants in the post-flowering stage develop more severe head blight at 30° than at 25°.

Clark and Dickson (20) discovered that the spot blotch phase of barley infected with *Helminthosporium sativum* reacted to a temperature series in different fashion from the rootrot- and seedling-blight phase caused by the same fungus. The spot-blotch development was most intensive at a temperature of 28°C., but symptoms developed quickly at a temperature of 24°, somewhat slower at 20°, and considerably slower at 16°. The rootrot and seedling blight developed over a much wider temperature range, and severe rootrot developed at 8° to 28° and maximum development usually occurred at 20°.

The very close relationship between certain environmental factors and infection of wheat plants by *Tilletia caries* and *T. foetida,* bunt fungi, has long been recognized. The exact effect of temperature and moisture on infection was strikingly illustrated by Kendrick and Purdy (70) in infection studies of the seed-borne inoculum of race T_5 of *T. caries*. Spore germination was equally good after ten days at 5°, 10° and 15°C. at soil moistures of 13 to 24 percent but declined markedly at higher temperatures. Although spore germination was 50 percent at 20° and 25° at the 18 and 24 percent moisture levels, infection dropped off rapidly at 20° at these same moisture levels. At 5° spores germinated well after ten days at soil moisture levels of 11 to 24 percent. However, infection was much lower at 5° than at 10° and at 15°, except at the ten percent level. Although spores of race T_5 can germinate over a relatively wide range of soil temperature and moisture, temperature is the critical factor in infection.

Severe epiphytotics of rice blast in June-planted rice have occurred several years during July and August in Belle Glade, Florida, when night temperatures averaged approximately 70°F. and the dew periods were 11 to 13 hours long. In general, the April-planted rice escaped infection, probably because of the low night temperatures during most of the period of leaf susceptibility. Greenhouse tests (68) showed that

infection with 3 races would subsequently develop into eyespot lesions over the range 60° to 95°. The minimal period of exposure required for infection at 80° was nine or ten hours; at temperatures below 80°, periods longer than ten hours were required for initiation of infection and the level of infection was lower than at 80°. After a 24-hour dew period, there were twice as many lesions on plants subjected to incubation in an 80°F. dew-cabinet as on plants at 70° or 65°

The seedling blight of smooth brome grass is the major factor in establishing field stands. *Pythium graminicola, P. irregulare* and *P. debaryanum* differ markedly in pathogenicity on brome grass seedlings as measured by total emergence and post-emergence blighting in sandy loam soil at 24, 20, 16 and 12°C. (157). Total emergence was reduced in soils infested with *P. debaryanum* at all soil temperatures but was lowest at 20° and 24°. Pre-emergence blighting occurred only at 20° and 24° in *P. graminicola*-infested soil and post-emergence blighting was also severe. In soil infested with *P. irregulare* at 20, 16 and 12° pre-emergence blighting occurred, but post-emergence blighting was not severe. *Fusarium roseum* and *F. oxysporum* did not cause serious injury under the conditions of the tests, and only a small amount of blighting after emergence was caused by *Rhizoctonia solani.*

The pre-emergence blighting and seedling injury is greatly influenced by the effect of temperature on the development of both host plant and the parasite. Leach (80) found that pre-emergence blighting and seedling injury were more extensive when crop plants were grown at soil temperatures that favored the growth of the pathogen more than the growth of the host. This temperature factor may explain in part, why post-emergence damping-off of brome grass was more serious at high temperatures since the optimum temperature for growth of *Pythium graminicola* and *Helminthosporium sativum* is about 28°C., whereas brome grass seedlings perform best at 20° and 16°.

The chief factor responsible for the predisposition of castorbean seed to pre-emergence damping-off was mainly planting immature seed. Thomas (155) noted that seeds that rotted without germination yielded *Pythium* spp., mainly *P. ultimum,* more frequently than all other fungi. Marked differences occurred among the varieties at temperature that gave maximum stand loss—10° for most inbred varieties; 20° for Cimarron, Illinois 224, Baker 43, and Baker 195. Hybrid varieties had poorer stands at 20° and 10° than at 30° or 15°.

Air temperature has a marked influence on the degree of resistance

of some barley varieties to stripe mosaic (135). At 16° to 20°C., resistant varieties were moderately to highly resistant in comparison with the susceptible Oderbrucker variety. At 24° to 28° all resistant varieties became moderately susceptible. Infected Oderbrucker plants usually failed to produce viable seeds at any of these temperatures. The resistant varieties yielded the maximum number of seeds at 20°; at 24° the seeds obtained were poorly developed and were few in number. None of the varieties produced seed at 28°.

When temperature conditions for plant growth were reversed by moving infected plants from 16°, 20°, 24° and 28°C. to 28°, 24°, 20° and 16°C., respectively, all varieties produced seeds in accordance with the final temperature conditions. In the temperature-reversal series it was shown that an initial period of 12 days at 28° allowed the virus to invade the seed even though the plants were returned to 16° for the remainder of the growth period. Temperature reversal at 20° and 24° had no noticeable effect.

Two hundred and six samples of winter barley seed affected with barley stripe mosaic developed the most pronounced disease symptoms at high light intensity and at 75° to 85°F. (56).

A change in the intensity of one weather element may bring about changes in the whole disease relationship. A change in temperature may make existing moisture conditions more or less favorable for attack by the pathogen or it may increase or decrease the vulnerability of the host.

The incidence of stem rust in the central wheat belt goes sharply up or down from high peaks in favorable seasons to low troughs in years when the disease is negligible or absent (95). Conditions for the development of stem rust epidemics are: *a*) mild winter weather permitting overwintering of uredospores in abundance as far north as Oklahoma; *b*) rapid build-up of the rust fungus; *c*) favorable weather for early infection in the southern portion of the central wheat belt; and *d*) continuing favorable weather along the way as spores are borne northward by the wind to produce infection in northern wheat fields.

During periods of high temperature or dry weather, many diseases cease progress and remain quiescent. When rainy periods occur, a disease may resume active progress. The incidence of *Phytophthora* disease in soybean plants is low in soil that has been air-dried for an extended period prior to planting. The appearance of the disease is

conspicuously higher if a prolonged wet period occurs before planting (74).

Climatic conditions extremely favorable for the elimination of bacterial and fungus diseases are ideal for the infection and development of curly top. This virus is transmitted by the leafhopper *Eutetix tenellus (Circulifer tenellus)* (133). After pasture vegetation on the plains and foothills becomes dry, the adults of the spring generation of hoppers fly into the cultivated areas and become abundant on cultivated plants, many of which are susceptible to the virus.

In areas where rainfall is about one inch during the growing season, irrigation has to be applied to supply the required amount of water. Water applied by furrow irrigation does not moisten the foliage to a great extent, and thereby provides favorable conditions for infection. Sprinkler irrigation of fields at first glance appears to simulate rain and to favor infection of fungus and bacterial diseases. In comparing sprinkler and natural rain there are several differences important to the spread of plant diseases. Natural rainstorms usually cover large land areas. They are usually preceded by a period of cloudy weather during which the temperature falls and the humidity increases. The rain is often accompanied by wind. After a rainfall there is a variable time of high humidity and cloudiness.

Artificial sprinkler rain is usually applied suddenly under hot, arid conditions and is abruptly terminated. The small land area is surrounded by hot, dry air which immediately moves in over the sprinkled field if there is any wind movement. Free water on crop foliage may be gone within 30 minutes after sprinkling ceases during hot weather, and the temperature drop is of short duration. These conditions are not inducive to initiate or spread fungus or bacterial diseases to an appreciable extent. If sprinkling irrigation is followed by rain and cloudy weather the change of environment would be favorable for development and spread of disease.

Menzies (92, 93, 94) reported some interesting observations on the occurrence of plant diseases during the first five years of the dry bean industry in newly irrigated areas of Washington. The assumption was that the newly developed diseases, such as *Sclerotinia* rot, bacterial blight, halo blight, bacterial wilt, rust and New York mosaic 15, originated from infected or contaminated seed.

In years of average rainfall none of the bacterial diseases was found in fields of furrow irrigated beans. Some disease was found in sprink-

ler-irrigated areas, and although the economic damage from bean blight under sprinklers was rare, there had been several border-line situations. A slight shift in the weather at a critical time might be enough to bring about a serious blight problem.

In 1953 mint rust *(Puccinia menthae)* was severe within an area covered by the sprinkler but was not found in the rest of the field until October. By this time a slight spread of rust to the leaves of most of the 40-acre field had occurred. Pod rot (*Botrytis* sp.) caused an exceptional amount of damage in a field under sprinklers. Count of 100 plants in eight locations in a 60-acre field showed an average of 18 percent of the pods infected. Moist soil contact was important, but in many cases very young pods were destroyed near the top of the plants.

Four bacterial diseases in field and lima beans were established by means of inoculations (94) in a furrow-irrigated field in central Washington. During the entire observation period, extending until September, no secondary infection from either pathogen could be detected. The same diseases were also established in a sprinkler-irrigated field. The initial inoculation areas were badly defoliated by bacterial blight, but no obvious infection was noted in adjacent rows. During a four-hour and eight-hour wet period, only a trace of infection was found on plants in a neighboring row. During a 12-hour and 30-hour wet period, secondary infections were more numerous, but spread to adjacent rows was only a trace. In the greenhouse secondary infection by the bacterial blight organism could be obtained (176). A thorough wetting of the plants prior or during inoculation is all that is necessary. The failure of 30 hours' continuous sprinkling to effect more than a trace of blight spread from heavily infected plants can be explained best on the basis of temperature rather than moisture.

In the Columbia Basin, sprinkler irrigation ordinarily does not favor bacterial diseases of beans enough for extensive damage to occur unless in an occasional year when unusual summer rains with accompanying lower temperatures could lead to bacterial disease development and losses in sprinkled beans (92).

Seed-borne *Phoma betae* is found in a number of sugar-beet seed lots in the United States. Germination tests of 125 seed lots showed heavy infection in several lots from the Willamette Valley, Oregon, where overhead irrigation was practiced, and only light to moderate infection in those from Medford, Oregon, Shasta Valley, California, and St.

Georgia, Utah. This discrepancy apparently can be explained on the basis of temperature relationship.

Raniere and Crossen (122) conducted detailed studies to obtain micromatic measurements of temperature, relative humidity, and dew period and intensity. On consecutive days following an irrigation, a series of measurements was made of temperature and relative humidity within the foliosphere of irrigated and unirrigated plots between the hours of noon and 6 P.M. This period is considered to be the most critical for spore survival.

Measurements of the RH at ground level on irrigated and unirrigated plots did not differ materially. These measurements of temperature and RH approximated the macroclimate six feet above the ground more closely than they did the microclimate within the plant foliosphere. The temperature in the plant foliosphere taken between noon and 6 P.M. on consecutive days following irrigation showed that differences between irrigated and non-irrigated plots did not exceed 4°F. However, differences in RH as great as 20 percent existed up to five days after a given irrigation. Average daily RH was consistently ten percent higher in irrigated plots than in the unirrigated plots. Between June 7 and September 1, free moisture, in the form of dew or rain, occurred on all but six nights in the unirrigated plots, and there were considerable differences in duration and intensity. The dew period in the irrigated plot was consistently longer than that in the unirrigated plots, averaging two hours up to six hours after irrigation. Dew-period differences as great as four to five hours were not uncommon.

Studies on the effect of moisture and RH on spore germination of *Colletotrichum phomoides* were conducted in the laboratory. These results showed that germination of freshly harvested *C. phomoides* was 90 percent or higher after six hours in sterile distilled water. *C. phomoides* germinated five to ten percent in the absence of free moisture at 93 percent RH, but only after a 48-hour exposure. Although spores germinated in an atmosphere of 100 percent RH, exposure to lower RH for periods of 12 hours reduced their subsequent ability to germinate. Spores exposed to 80 percent RH and then transferred to free moisture exhibited about 15 percent germination after 120 hours. Twelve-hour exposures below 80 percent RH apparently were fatal, indicating a great sensitivity of the spores to desiccation.

In view of the sensitivity of spores of *C. phomoides* to desiccation.

the longer and more intense dew periods in irrigated plots are thought to be important in increased incidence of tomato anthracnose. The initial effect of overhead irrigation may be dissemination of inoculum. The more important effect seems to be increased RH and longer dew periods. During average or drier-than-average periods, rain might very well disseminate inoculum in unirrigated plots that is not viable because of exposure to low RH; on the other hand, inoculum disseminated in irrigated plots, whether by splashing rain or overhead irrigation might be highly viable.

Additional information in determining how overhead irrigation influences microclimate and disease development of various other crop plants should be obtained. With such information a more intelligent scheduling of the application of overhead irrigation should be possible.

The producers engaged in the development of disease-free seed are keenly aware of the importance of environment in causing disease and select areas for the production of seed where climatic conditions are unfavorable for initiation of disease and its subsequent development.

As a result, vegetable-seed production areas have been shifted to areas where the weather is less favorable for the development of plant diseases. Before 1925 the production of bean seed was limited to the New England-New York area and Michigan. Bacterial blight and anthracnose were widespread and destructive. The causal organisms of these diseases are disseminated chiefly by the seed. Bean seed is produced now mostly in Idaho and California where exceptionally low humidity prevails and little hail occurs in these states during the growing season. Under such climatic conditions these diseases do not develop.

Pea seed for gardens and canning was formerly grown primarily in the northeastern States. Pea seed is now produced in the Snake River Valley, the Twin Falls area of Idaho, the Palouse section of northern Idaho, eastern Washington, and in the Imperial and Temecula Valleys of California. The shift in location was made because the climatic conditions prevailing in these areas are favorable to the production of pea seed free of bacterial blight *(Pseudomonas pisi)* and leaf spot (*Ascochyta* sp.)

In the Imperial and Temecula Valleys of southern California (172), naturally infected and artificially contaminated pea seed of the varieties Little Marvel, Alaska, and No. 76 produced seed free from infection by *Ascochyta* sp. In the Temecula Valley peas are grown during the

dry summer period. In this area the average total rainfall from August to the end of December is 1.2 inches and from November to March 1.64 inches.

Cabbage, turnip, rutabaga and cauliflower seed were produced in the middle West and the East at one time. On account of the ravages of blackleg *(Phoma lingam)* and blackrot *(Xanthomonas campestris)* there, the seed-producing areas are now located along the Pacific coast. Low rainfall during the time the seed plants are grown prevents these two diseases from becoming established.

In the southern part of Louisiana (158) the growing of onion seed had to be discontinued because of excessively humid conditions, stalk rot diseases, mildew, purple blotch and development of black rot chiefly during blossoming and seed-setting time.

Anthracnose and bacterial blight cause the greatest losses in snap beans. On account of the almost rain-free summers these diseases do not occur on bean seeds grown in California (86).

In some countries it is not possible to find areas of such restricted rainfall to produce disease-free seed. The most important seed-borne diseases of beans in New South Wales, Australia, are halo blight *(Pseudomonas medicaginis)* which affects most varieties of dwarf French beans, and American common blight *(Xanthomonas phaseoli)* on navy beans (172). It is recognized that disease-free seed can be produced in areas having less than one inch of rainfall in the growing season, but there are no areas in New South Wales where the summer rainfall is low enough. The problem of obtaining clean seed of susceptible varieties cannot be solved in New South Wales by any reliance on dry conditions to prevent spread, but is solved rather by a certification scheme with zero tolerance.

FIELD SANITATION

The most effective way to produce disease-free seed is to start out with healthy seed and to prevent infection from developing during the growing season. If infection does occur, disease spread can be reduced by crop rotation, seed treatment, spraying or dusting, isolation, roguing and disinfection of containers.

Since a number of disease-producing organisms live over in the soil, it is advisable to rotate with non-susceptible crops to eliminate or reduce the infecting organisms by starvation. Some of the organisms

producing disease can remain in the soil extended periods. Some disease-producing organisms that live in the soil are not eradicated by crop rotation, but the amount of infection may be greatly reduced by this method.

Seed treatment aids in destroying spores and other forms of pathogenic organisms that appear on the surface of seed, and also in ridding the seed of a pathogen which has penetrated and become established within the seed.

To control some leaf diseases, spraying or dusting must be done before the disease appears. The foliage should be thoroughly covered with the fungicide. When spores of a leaf-disease fungus drop on spray-covered foliage, the toxic substances of the sprays will kill the spores before they germinate and invade the plant.

Isolation is also a method of disease control. Crop plants should be isolated from similar crops infected with disease and also from other alternate host plants including weeds.

Roguing is generally used more to control virus diseases than those caused by fungi or bacteria. The sole purpose is to remove infected plants from a field to prevent spread of the disease to surrounding plants in the same field.

Control of potato ringrot *(Corynebacterium sepedonicum)* lies in its complete eradication from individual farms (119, 147). This entails proper disposal of all potatoes, infected or clean, on the farm where ringrot has been found. Storage places, machinery, tools, bags, etc., should be cleansed and disinfected because the ringrot bacteria may be carried on burlap bags for seven months whether stored inside or outside a cellar. Only clean seed potatoes should be planted the following season.

The cull pile has been found to be a source of inoculum of the late blight fungus *(Phytophthora infestans)* (13). Plants growing on the cull pile have been observed to be infected on Long Island and Maine early in June. The disease may spread from the cull pile to adjacent fields.

Soils in which potatoes are grown are often contaminated with *Fusarium caeruleum,* the causal organism of dry rot (137). Contaminated seed boxes may be a source of infection, especially if the tubers are bruised. The small losses among washed, bruised tubers show that little, if any, infection generally occurs before digging. In one test with 500 freshly dug tubers, removal of the soil by washing reduced

dry rot from 20.7 to 3.1 percent and in another from 10.4 to 0 percent. Mature wounded and unwounded tubers became infected when placed in contact with contaminated soil.

Additional studies revealed that seed tubers which had not been bruised during digging, whether disinfected or not, were practically sound after 6 months' storage. Severe infection by *F. caeruleum* developed during storage in tubers not previously dipped and deliberately bruised. Apparently healthy tubers, even if taken carefully from boxes contaminated with *F. caeruleum,* became severely infected.

Potato wart disease *(Synchytrium endobioticum)* became established in this country in 1911, mainly in Pennsylvania. An eradication program in Pennsylvania got underway in 1932 to rid the State completely of the wart organism (57, 169). Gardens and infected fields were spaded or plowed after removing all weed growth and rubbish and were treated with either ammonium thiocyanate or copper sulphate at the rate of 2500 to 3000 pounds to the acre. The area was then check-planted for susceptible potato varieties for four years. If no wart was found, the ground was considered free from the wart organism.

A check planting of ten-year-old infested areas, which had been kept under continuous garden culture, but free from susceptible host plants, showed that wart had persisted in a small percentage of infection sites. Similar checks of areas kept in sod showed a 100 percent infection. At the end of the 15th year, the fallow, vegetable, and wart-immune potato units were wart-free, whereas 50 percent infection was evident in the sod units. By the 20th year all units were free from potato wart.

The curly top virus affects many cultivated crop plants and is of considerable economic importance, especially on the Pacific Coast. Russian thistle is a joint host of the curly top virus and its leafhopper vector, *Circulifer tenellus.* To destroy this host in 1950, 564,000 gallons of insecticide spray were applied in California between October 1 and November 1 to 141,000 acres. As a result, the average percentage of infection on tomato in seven counties in San Joaquin Valley was reduced from 43.8 in 1950 to 5.2 in 1951; the corresponding figures for Monterey were 31.1 and 2.3. The over-all incidence of infection in the areas protected by spraying was estimated at 0–30 percent compared with a normal range of 36 to 100 in similar untreated areas. In the spring of 1950 the losses incurred by growers of tomatoes in certain critical zones approximated 100 percent, whereas in 1951, after spraying, the incidence of infection was well below ten percent and a full crop was harvested.

The average percentage of curly top in late tomatoes was less than one as against a normal expectancy of 20.

To eradicate effectively the overwintering hosts of the vector of a virus responsible for losses amounting to millions of dollars, it is necessary to conduct a very extensive eradication program. This requires state legislative action. In 1950 and 1951, $350,000 had to be expended for the emergency measures against Russian thistle.

Analysis of 852 weed plants belonging to 42 species and collected from sites where they had a good opportunity to be infected by the virus of beet yellows showed that 25 plants in all of *Chenopodium bonus-henricus, Senecio vulgaris, Sonchus oleraceus* and *Stellaria* sp. contained yellows virus while 38 species did not. *C. bonus-henricus,* not formerly reported as susceptible, retained the virus in the field for at least two years (11). Infected plants of the winter-annual species were found fairly often, and in a mild winter may act as a significant virus reservoir in addition to those already known.

Buckwheat, rape, radish, clover, vetch species, flax, carrot, *Chrysanthemum leucanthemum,* other chrysanthemum species, and salsify were among the plants susceptible to the potato yellow dwarf virus (175). In the field *C. leucanthemum* was commonly infected with the virus, but only half of the infected plants showed symptoms. This variety overwinters and provides a source of virus the following spring.

In West Friesland, Netherlands, where carrots are grown extensively for seed production, *Phomopsis dauci* is responsible for losses of up to 80 percent of the umbels (9). Plants growing in cheesecloth-covered cages escape the disease and remain healthy. Spraying with Bordeaux mixture and other fungicides at ten-day intervals after the appearance of the first flower buds gave promising results. To reduce celery seed infection by *Septoria graveolentis,* heavily infected plants were given two Bordeaux sprays from November to December and from April until 14 days before the seed was harvested (39). This treatment reduced seed infection to 0.5 percent.

Seed of cabbage, turnips and swedes for the production of seed in the Puget Sound area in Washington is sowed in early June and July (117). Transplanting begins about August 1, and the seed is harvested during July and August of the following year, so the culture allows no cabbage-free period during which disease organisms can be destroyed. In the summers of 1943, 1944 and 1945 the disease indexes for cabbage mosaic were 67.5, 68.9 and 44.6. Widespread infection

resulted from the nearness of the seed beds to diseased fields. Beginning with the 1944-45 crop, several cabbage seed beds were grown in isolation, and approximately 60 percent of the transplants set for this crop came from these. There was a marked drop in the mosaic index for 1944 and 1945.

The disease indexes for downy mildew *(Peronospora parasitica)* were 47, 53, and 41 in the summers of the three years. This disease usually causes more serious losses in the seed than in the head stage. Fields kept under clean culture, properly staked and twined, were consistently less seriously infected than poorly kept fields.

"Hoja blanca," a virus disease of rice, has been discovered on rice at Belle Glade Experiment Station, Florida. A 40-acre field adjacent to the station and volunteer rice some five miles west of Belle Glade were also affected. Barnyard grass (*Echinochloa colonum* or *E. crus-galli*) and the weed *Panicum fasciculatum* also appeared to show evidence of the disease (6). All infested areas were immediately sprayed with an insecticide, affected rice ploughed under and infected grass destroyed with chemicals.

The most destructive disease of soybeans, especially of the yellow variety in Germany, is mosaic (60), which necessitates stringent field sanitation to prevent perpetuation of the virus in the seed of infected plants. The first count, four weeks after sowing, revealed an incidence of 23 percent diseased plants, which rose sharply during the summer owing to an exceptional prevalence of aphids. There was a marked difference in spread between the plots from which infected plants had been eliminated and the untreated controls.

All the commercial lettuce seed lots tested in California contained from one to three percent lettuce mosaic virus. Several pounds of mosaic-free seed of the Imperial 615 and Great Lakes varieties were produced by growing plants in an insect-free greenhouse and roguing out all that were infected before transplanting to a mosaic-free locality. Several test plots, surrounded by commercial lettuce, were sown with samples of the mosaic-free seed in the Salinas Valley region in the spring of 1951. The percentage of infection in these test plots was substantially reduced, indicating the practicability of combating the disease by this method.

The effects of roguing commercial lettuce crops affected by mosaic virus were determined by (*a*) cutting the plant off with a hoe and leaving it in place; (*b*) oil-roguing, i.e., killing the plant with a mixture

of contact herbicide and insecticide; and (*c*) roguing the affected plant and one plant on each side in the row by methods (*a*) and (*b*). The first roguing was done seven to 15 days after thinning; in some plots a second roguing was done seven to ten days later, and in others a third after the same interval.

A single roguing by any of the methods did not significantly decrease the percentage of infection at harvest; roguing twice, however, decreased it significantly (177). The greatest reduction resulted from three roguings with oil (5.8 percent infection as against 17.2 percent for the unrogued), but three hoeings were almost as effective (10 percent infection). The reduction in infection resulting from use of mosaic-free seed was much greater than that from any roguing method.

Since practically all potato virus diseases are transmitted through tubers, in the production of seed potatoes an important factor is to remove as nearly as possible all sources of virus infection.

The following methods are used to eliminate virus diseases from seed stock of potatoes: (*a*) tuber indexing, (*b*) planting in tuber units, (*c*) mass roguing, (*d*) hill selection, and (*e*) early harvesting (42).

Tuber indexing is the most effective method of eliminating diseased potato tubers from seed stock intended for planting in the seed plot. It involves numbering all tubers and removing from each a seed piece containing one eye. This seed piece is given the same number as the tuber from which it was taken and is planted well before field planting to determine whether the tuber is healthy. All tubers detected as diseased by the performance of plants from the indexed pieces are discarded, and only the healthy ones are kept for planting in the plot.

In the North tuber indexing is limited to the greenhouse. However, it is possible for northern potato growers to practice outdoor tuber indexing by sending the indexes from the numbered tubers to southern New Jersey, the Eastern Shore of Maryland, Virginia, or practically any other southern State, to be planted in the open field after being properly labeled. By having such work done in the South, it is possible to grow the indexed set and take readings of disease symptoms before the normal planting time in the North. At the usual planting time the tubers found to be free from disease are planted as tuber units in a seed plot. It is then easy to identify diseases in the plant growing from infected tubers that may have escaped observation previously.

There are several satisfactory ways of planting the tuber in units. All sets from each tuber should be planted together in a row, so

separated from the sets of the next tuber or so marked as to permit ready and accurate recognition of each tuber. A common practice is to select from the bin of seed potatoes well-shaped tubers ranging from six to eight ounces. These are cut into four pieces and planted about 12 inches apart in the row. One space is left unplanted so that there is a two-foot space between the units. Units thus spaced can be identified accurately during growth and all the plants can be removed if any in a unit is affected with a virus disease. If one seed piece produces a plant affected with a virus disease, it is best to remove all the seed pieces from that unit, even though some of them may not be up, for usually all are affected. Planting the plot in a tuber unit enables the grower to recognize diseased plants more readily and permits roguing earlier and more accurately than is possible with other methods. It is easier to detect disease when the seed pieces can be removed before they are up if the more advanced sister hills in the same tuber unit show disease symptoms.

In mass roguing of seed plots, the tubers are planted at random, either as whole or as cut seed. As soon as the plants are high enough so that the disease symptoms can be recognized, the diseased plants and the seed pieces are removed. As soon as these plants are pulled, they are placed in a sack or other closed container to prevent escape of disease-carrying insects and are disposed of at a distance of at least 500 feet from the seed plot. Effective roguing by this method has proved to be very difficult. Experiments have shown that in the tuber unit plot practically all the diseased plants are removed in the first roguing, whereas in the mass plot many individual plants are too small to show disease symptoms and cannot be removed before the second or third roguing.

Selecting only apparently healthy hills from a field where virus diseases are present may result in reducing diseases to some extent the following year, but this method is entirely inadequate as an effective means of controlling virus diseases of potato. In a test, one tuber from each hill selected as healthy was grown as an index before field planting. It was found that 15 percent of plants in hills that looked healthy were definitely infected with rugose mosaic. These hill lots were discarded, but when the hills, supposedly healthy, were planted in the field, 8.5 percent of the plants were still infected with rugose mosaic. An explanation is that when a plant is infected late in the

season, no symptoms may be evident; part of the tubers of such a plant may be diseased and the remainder healthy.

Control by early harvesting consists in killing or removing the tops and digging the tubers early in the growing season before viruses that are introduced into the foliage by insects have had time to reach the tubers. Experiments in Maine showed that when potatoes were harvested between July 25 and August 7, no spread of viruses took place in six of nine years, and the spread amounted to less than one percent in the other three. In 1940 only 0.8 percent of the tubers harvested in July had virus diseases; but of 35 percent of those harvested from the same field in September, 20 percent of these plants had mosaic and 33 percent had leaf roll.

Some states have found it advantageous to provide a roguing service directed by the State Agricultural Experiment Station for the service of the growers of foundation seed (134). The experiment station undertakes to employ roguers and supervise their work. The roguers have to detect and remove diseased units. Among the chief factors in successful handling of foundation seed plots are *a*) a source of seed known to be free from bacterial ring rot, *b*) early planting by tuber units, *c*) isolation, *d*) thorough roguing *e*) early harvesting, and *f*) storage on the farm where contamination of the seed stocks will not occur.

STORAGE

Storage is an exceedingly important phase in maintaining seed free from disease and possessed of high viability. An understanding of the significance of all factors that may operate to affect the physiological and pathological changes of seed in storage is an essential part of a complete program to produce disease-free seed. These storage factors include: *a*) use of proper bulk storage or of individual containers; *b*) the maintenance of correct temperature and moisture conditions for control of molds; and *c*) the use of fumigation practices to control weevils and virus vectors in storage.

Yellow dent shelled corn stored in steel bins of 1000- to 2740-bushel capacities showed 50 percent mold infection in August and by the following April the infection had risen to 73 percent (132). During the winter months the most prevalent fungi on the corn were *Penicillium palitans* and *Aspergillus candidus,* mixed with species of *Mucor,* other

species of *Penicillium* and *Aspergillus,* and *Rhizopus absidia.* Corn stored in tight metal containers at 20° to 25°C. showed nearly 50-percent germinability loss after one year when kept at 14-percent moisture content with complete loss after two years. At 9.7-percent moisture the corn showed no change during the same testing period. A relative humidity of 65 percent appears to be the lower limit for mold growth in corn.

It was definitely shown that, of 159 samples of corn stored commercially for 6 to 9 months, those with the poorest germination had the highest percentage of fungal infection, especially by *Aspergillus flavus* and *Penicillium* spp. (118). Low temperature checked damage as effectively as low moisture. Corn at harvest time is generally free from infection by the fungi subsequently involved in storage rots, and germ damage apparently results entirely from micro-organisms in storage and not from any process inherent in the seed.

During a 20-days storage period the seeds of Regent wheat showed no significant increase in the incidence of fungal infection when moisture content was below 14.5 percent, RH 75 percent (97, 98). In this same period the seeds underwent no chemical or germinative deterioration. However, as moisture values advanced beyond this critical zone, respiratory rates increased. Similar increases were noted in chemical deterioration, moisture content of the seed, seed devitalization, and mold growth. Prevalent molds were *Aspergillus glaucus, A. candidus, A. flavus* and *Penicillium* sp. Extent of mold growth was directly related to the respiratory activity and amount of chemical and germinative deterioration of the grain. In damp wheat samples the temperature and respiratory increases are directly correlated with mold growth up to 52° to 55°C., at which temperatures the fungi are killed, respiration is inhibited, and heating ceases.

When wheat was inoculated with *Aspergillus candidus, A. repens* and *A. restrictus* and stored up to seven months in desiccators at 25°C. with moisture content varying from 14.7 to 20 percent moderate reduction in germination occurred (113). At moisture content of 14.7 to 14.9 percent *A. restrictus* was more deleterious than the other species. At 15 to 16 percent moisture content *A. candidus* caused a rapid decrease in germination and abundant sickness. The heavy contamination of commercial samples by these fungi implies that these species are seriously implicated in the deterioration of seed.

Marked differences occur in the rate of deterioration of different lots

of wheat stored under essentially the same conditions. These variations may be due in part to the degree to which the different lots of wheat have been invaded by storage molds before they are stored (174).

Wheat at times is stored in bulk at 35° to 40°C. and at a moisture content of 15 percent. Storage under such conditions for six weeks or more might result in extensive invasion by storage molds and a great decrease in germination. From the mycological standpoint such grain would be considered to be partly deteriorated although this deterioration would not be detectable by the ordinary inspection procedures used to determine the quality of grain.

Freshly harvested grain is often free from all but light and superficial infections by storage fungi. Even upon arrival at terminals it usually has not been invaded by storage molds. Subsequent invasion by fungi may lead to extensive damage before any trouble becomes evident from the outside appearance of the kernel. Tests to determine the number and kinds of storage fungi present in the kernel and the increase of these fungi during the storage period are now used by some operators as a regular part of storage operations.

The dark germs, or embryos, typical of sick wheat have been found in samples stored 10 weeks at 40°C. and moisture content of 16 and 17 percent. In nearly all these, no fungus is visible on the outside of the kernel, but the embryo is overgrown and the cavities within the embryo are filled with mycelium.

The actions of weevils in stored grain on increasing the moisture content and amount of fungal infection of seed were determined in recent studies (1). Replicate samples of hard red spring wheat infested and uninfested with *Sitophilus granarius* were stored for one to three months at 25°C. and 75 percent RH. Increases in the insect population were marked by rises in the moisture content and amount of fungal infection.

Hyphae and germinating conida of *Aspergillus restrictus* and others of the *A. glaucus* group, including *A. repens, A. amstelodami* and *A. ruber,* were detected in the intestines of the insects and recovered from their excreta. These persisted until after death of starved individual insects. When only surface disinfected weevils were added to clean grain prior to five weeks at 85 percent RH, the number of colonies of *A. restrictus* rose to over 5,000,000 by the end of the test. An increase in moisture content from 14.9 to 20.8 percent was recorded. The fungus was absent from insect-free control samples.

One hundred mold-inhibitor compounds were tested for their efficiency on wheat (98). Of the two most effective preparations, thiourea was only slightly toxic to seed stored with moisture content below 24 percent, but 8-hydroxyquinoline sulphate reduced germination by over 30 percent. Sound wheat, stored at 30°C. with moisture content above 16.1 percent, was rapidly overgrown by molds. The increase in respiration and decrease in viability of the seed with rising moisture content were proportional to the increase in mold incidence. Wheat treated with one part of thiourea to 100 parts by weight of moist seed respired at a nearly constant rate over a ten-day period with only a slight decrease in viability and a small increase of molds up to a moisture content of 21.3 percent. At moisture contents of 27 to 35.5 percent, the viability of the seed was reduced. The fat acidities of wheat seed treated with thiourea were markedly lower than these of the untreated when held ten days at 30°C., especially at moisture contents above 18 percent.

The respiration of dormant seed on which molds were inhibited, but not eliminated, increased gradually with a rising moisture content until the process involved in germination began to operate. The sharp increase in respiration of the seed at the so-called critical moisture content is caused by the respiration of molds on and in the seed.

It was shown that the time taken for deactivation of parasites on flax seed in storage is usually much longer than that at which flax seed for sowing purposes can safely be stored (24, 130). The parasites involved were *Colletotrichum linicola, Polyspora lini, Botrytis cinerea* and *Phoma* sp. The longevity of the fungi varied from 26 to 69 months, 16 to 55 months, 16 to 40 months, and 27 to 43 months, respectively. The germination capacity of the seed was usually seriously impaired by the time all the parasites had died.

Tests with cotton seed in storage confirmed the results of previous tests with wheat, corn and flax seed, that molds including *Penicillium* sp., *Aspergillus glaucus, A. ochraceus, A. candidus, A versicolor, Glomerella gossypii* and *Fusarium vasinfectum* may play an important part in the deterioration of moist, stored cotton seed.

The aphid *Myzus persicae* was capable of acquiring the tulip-breaking virus from the colourless sprouts and bulb scales of *Lilium longiflorum* bulbs in storage and of transmitting it to similar sprouts of Easter lily seedlings, (138). Cucumber mosaic was readily transmitted by *M. persicae* from growing Easter lily to growing plants or sprouts

of Creole lily in storage. *Aphis gossypii* transmits the lily rosette virus from sprout to sprout or from sprouts to growing Easter lily plants. This aphid can transmit the virus during storage and carry it to growing plants in adjoining fields. Both persistent and non-persistent lily viruses are communicable during storage of the bulbs by the appropriate insect vectors.

It is well known that *Myzus persiae, M. pelargonii* and *M. circumflexus* can transmit potato viruses from sprouted tubers in storage.

Late blight *(Phytophthora infestans)* spread from infected to healthy tubers in seed bags (44) when the potatoes were stored out-of-doors and remained wet for prolonged periods during rainy weather. Infection also spread from infected to healthy tubers when tubers were kept in continuous shade after a rain. This latter procedure caused the tubers to dry slowly. The development of the pathogen was favored by 25 rainy days in December and January. Fifteen percent of the seed exposed to this rainy weather contracted infection. When the seed was later planted in Florida, the fungus made rapid growth in the seed pieces and formed spores on the sprouts and young stems. Several of the infected seed pieces died or produced weak plants.

Storage of potatoes involves greater complexities than that of any other crop: (19, 42, 44, 53, 84, 114, 123, 141, 151). Potatoes are alive and composed of considerable fleshy tissue. Therefore, they carry on within themselves several of the processes characteristic of all living things. Respiration causes a loss in weight, but the principal shrinkage of potatoes aside from rot is from transpiration, or loss of water. Poor quality potatoes shrunk in weight during a six-month period in cold storage about twice as much as the U. S. No. 1 potatoes.

Diseases of potatoes developing during storage are of two kinds: *a*) those that originate in the field, and *b*) those that originate after harvest owing to the entrance of disease-causing organisms following mechanical or heat injury to the tuber In the first category are leak, *Alternaria* tuber rot, late blight tuber rot, and physiological troubles, such as freezing and blackheart.

The soft rot bacteria can enter potatoes through cuts, bruises, skinned places, or other mechanical injuries. Heat-injured potatoes are more subject to soft rot than normal tissues (43). Even temperatures after digging, not high enough to kill the tissues, make them susceptible to rot. The effect of such temperatures is reversible and may be corrected to some extent by subsequently keeping the potatoes at a lower temperature.

Heat damage may be expected whenever potatoes are exposed to the sun for 15 minutes or longer on days when the air temperature is 90°F. or higher. The potatoes absorb the heat rays of the sun and may develop surface temperatures 25° to 40° higher than the surrounding air. Heat damage is likely to occur when the surface temperature is 110° or higher. Potatoes should be removed from the field before they are exposed to the heat of the sun, and stored at temperatures below 70°.

Improper storage conditions may bring about blackheart, a physiological condition. Blackheart may develop at 70°F. if the oxygen supply is reduced. It also develops at high temperatures regardless of the oxygen supply because of the inability of the potatoes to absorb oxygen fast enough to supply the rapidly respiring internal tissues. The internal tissue turns black and is usually at the center of the potato, sharply differentiated from the healthy.

The skinned areas of potatoes may turn brown when subjected to dry currents of air. The browning is accompanied by a loss of moisture which results in withering and shrinkage of affected tissues. It is apparently associated with too rapid drying of injured tissues. This rapid drying prevents the formation of wound periderm. Browning does not occur if potatoes are exposed to high humidity and temperatures favorable for wound healing for 12 hours after they are skinned.

One of the most important problems in reducing storage losses of potatoes is the problem of injury of the tubers before they are placed in storage. Sometimes the injury is so prevalent and so severe that it is impossible to sort the stock to meet any of the better grades. It is necessary to impress on those who do the physical handling the fact that, unlike coal or gravel, potatoes cannot be roughly handled.

PLANT QUARANTINE

To maintain disease-free plants it is necessary not only to control and to eradicate diseases but also to prevent introduction of diseases not as yet found in a country. Modern commerce and world economy are conducive to an international flow of plants and products, together with plant diseases which these materials may often carry.

An effective way to prevent introduction of new plant diseases is by quarantines. A quarantine is a restriction imposed by duly constituted authority, whereby the production and the movement of plants or plant products are brought under regulation so that the spread of a pest may be prevented or limited.

In the United States the Federal Plant Quarantine Act provides many safeguards against entry or establishment of foreign plant diseases as well as against the domestic spread of introduced diseases (21, 35, 89, 128, 170).

For domestic commerce, the Act gives the Secretary of Agriculture authority to regulate the movement of anything which could carry a dangerous plant disease. The Secretary may quarantine any State, territory, or district of the United States, or part thereof, whenever he determines it is necessary to do so to prevent the spread of a new dangerous plant disease.

The importance of a pest in the national sense depends somewhat on the threatened host. A pest affecting a basic crop such as corn, potatoes, or apples is far more significant than if it attacked hosts like rhubarb or parsnips. Host range is another criterion. Also, a pest attacking a wide range of cultivated hosts may rate a much higher listing than a similar pathogen restricted to a single host. The possibilities for biological control and the success of resistant varieties are aspects which may profoundly affect the overall importance of a pest at times. In gauging the national status of a pest as a basis for quarantine action, all above-mentioned factors may be called in to contribute to the appraisal. A great many disease situations have come up for quarantine consideration. Federal quarantine powers have not always been invoked for these for a number of reasons: *a*) the disease may be too unimportant nationally to justify quarantine effort; *b*) quarantine usefulness is questionable because of the recognized inadequacy of available measures in preventing spread; *c*) the level of national support may be inadequate by popular apathy, adverse influential sentiment, or the clash of conflicting interest.

Without quarantine barriers, the United States could readily become the habitat for diseases not now known in this country. During the 12-month period ending May 21, 1956, some 84,000 aeroplanes and 46,000 ocean vessels arrived from abroad. Plant quarantine inspectors also checked 10,000 planes in Hawaii and 2,300 in Puerto Rico before departure of the aeroplanes for the mainland. When inspected, 37,000 of the planes were found to carry prohibited or restricted plant materials. Inspection activities at seaports yielded 244,500 lots of contraband material.

The Agricultural Research Service receives information from foreign countries on pests that are indigenous to them and on outbreaks that

reach dangerous proportions. This information enables port plant quarantine inspectors to know what to expect from materials coming from different parts of the world.

During recent years the Plant Quarantine Branch has sent inspectors to foreign countries to inspect the packing of plant material for shipment to the United States. Such "Country of Origin" inspection helps prevent entry of diseased plant material.

When a new crop is introduced into a country, some of the native pathogens may attack it. While new crops are being bred, including those bred for disease resistance, it should be kept in mind that various pathogens also may be developing new races or finding new susceptible hosts. Introduced pathogens also may attack native crops in an unsuspected way. Inspection of the growing crop in a foreign country before importation has been a big step forward for all concerned.

To keep this program effective, disease surveys have to be made so that any incipient incidence of a pathogen may be detected while the area involved is small and controllable. Frequently, these surveys provide an opportunity for eradication at reasonable cost and without serious impact on the industry.

The Agricultural Research Service also evaluates information on pests in this country. For example, the Plant Disease Warning Service reports the intensity and extent of plant disease incidence. As an illustration, a Plant Disease Warning Service letter went out telling of the appearance of the soybean cyst nematode in western Tennessee, some 400 miles from North Carolina, the only other State in which it had been reported.

The golden nematode *(Meloidogyne rostochiensis)* presents a current problem of wide interest and of a potentially serious nature. It is recorded as occurring on the potato in Germany in 1881. Other European records indicate its presence in Scotland, England, Ireland, Sweden, Denmark and the Netherlands. In Europe it has notably damaged potato production.

The golden nematode was first found in North America in 1941, associated with low yields in a few Long Island, New York, potato fields. Intensive surveys there indicate that the soil in about 8000 acres of potato lands around the original center is more or less infested with this potato root parasite. Considerable search in other potato-producing States has failed to disclose this nematode elsewhere.

Theoretically, quarantine action against a pest of this type would

call for a federal interstate quarantine, supplemented by parallel action by New York State. Because of the isolated character of the infested center on Long Island and the measures that New York would be forced to adopt to protect its other important potato districts, it is believed that the State quarantine could provide he protection necessary for the interest of other States as well as its own.

Absence of federal quarantine connotes no lack of federal interest in the nematode problem. Federal agencies have cooperated to the fullest extent in matters of research, survey, control, soil treatment, and the enforcement of plant quarantine regulations.

A good illustration of an effective plant quarantine that has met with popular support is that of the black stem rust *(Puccinia graminis)* of cereals. This disease has been in the United States since early colonial days. The causal organism is spread rapidly by wind. Because of all this, quarantine would appear to be useless, yet quarantine action has a definite place in the stem rust situation because of the important role of the alternate hosts, *Berberis, Mahonia* and *Mahoberberis,* in starting early local infections and in serving as a breeding ground for new and more virulent stem rust races. Quarantine effort aims to prevent the movement of rust-susceptible barberry and *Mahonia* into grain-growing areas and to prevent replanting of these undesirable rust hosts in areas already cleared of them.

SEED CERTIFICATION

The purpose of seed certification is to maintain and make available to the public sources of high quality seeds and propagating materials of superior varieties of known genetic identity. Certified seed is high in varietal purity and of good seeding value (5). Varietal purity is the first consideration in seed certification, but weed seeds, disease-producing agents, viability, mechanical purity, and grading are also important. Seed certification is designed, therefore, to maintain not only the genetic purity of superior crop varieties but also reasonable standards of seed condition and quality.

Production of certified seed of superior varieties of field and forage crops involves the cooperative effort of many State, federal, and private agencies. Among these are State agricultural experiment stations, extension services, State departments of agriculture, State crop improvement associations, the International Crop Improvement Association, the seed trade, and the United States Department of Agriculture.

The organization or agency assuming certification should be designated under State or national legislation and have a close working relation with seed growers and agricultural research, extension, and seed-regulatory agencies.

Four classes of seed are recognized in seed certification, namely, breeder, foundation, registered and certified. These classes must meet the standards of the International Crop Improvement Association for the respective crops. They are defined as follows:

BREEDER SEEDS. Seed or vegetative propagating material directly controlled by the originating, or in certain cases, the sponsoring plant breeder or institution, which provides the source for the initial and recurring increase of foundation seed.

FOUNDATION SEED. Stocks that are so handled as to maintain most nearly specific genetic identity and purity and which may be designated or distributed by an agricultural experiment station. Production must be carefully supervised or approved by representatives of an agricultural experiment station. Foundation seed shall be the source of all other certified seed classes either directly or through registered seed.

REGISTERED SEED. The progeny of foundation, registered, or certified seed handled so as to maintain satisfactory genetic identity and purity and approved and certified by the certifying agency. This class of seed should be of a quality suitable for the production of certified seed.

CERTIFIED SEED. The progeny of foundation, registered, or certified seed that is so handled as to maintain satisfactory genetic identity and purity and approved and certified by the certifying agency.

In cases where the seed planted for the production of foundation, registered, or certified seed is obtained from another person, documentary evidence, such as certification tag and sale's record, must be submitted to the certifying agency to establish the source of seed.

Every field for which certification is requested shall show evidence that reasonable precaution has been taken to control seed-borne diseases. The field at time of inspection shall not contain any diseases beyond the established tolerances of injurious seed-borne plant diseases which are enumerated in the individual crop standards. The representative samples of the finished seed crop should be subjected to laboratory examination for diseases.

One or more inspections of harvested seed lots from inspected fields may be made at any time by representatives of the certifying agency

who shall have authority to reject for certification any lot not properly protected from mixture or disease. Analyses and tests of seed samples and definitions of analytic terms shall be in accordance with the rules of the Association of Official Seed Analysts.

All seed stocks, when sold as certified seed, shall have an official tag properly affixed to each container. The certification tag which is attached to the bag serves as evidence of both the genetic identity and purity of the seed contained therein. The regular certification tag may be used for identifying foundation and registered seed. The word "foundation" or "registered" may be printed or stamped on the regular certified seed tag for seed that meets the requirements of the foundation or the registered seed class.

If other than regular certified seed tags are to be used for "foundation" or "registered" seed, they should be white for foundation seed (and breeder seed, if tagged) and purple for registered seed.

The quality of the certified class should be designated as blue tag for first quality seed and red tag for second quality seed.

In meeting certification requirements for vegetable seed or other field-crops' seed, the major emphasis is placed upon freedom from mixtures of other varieties, objectionable weed seeds, inert matter, split and cracked seed, discoloration, germination, and hard seed rather than upon diseases affecting quality or transmissible through seed but these are considered.

In seed-potato certification, purity of variety is required, but the major emphasis is placed upon freedom from diseases. Since potatoes are propagated asexually, most of the diseases affecting potato plants can be transmitted through the tubers. The tremendous expansion of potato culture throughout the world during the last half of the nineteenth century was accompanied by the dissemination of many of the more serious diseases of the plant (3, 116). During this period, potato seed stocks were freely exchanged on a world-wide basis, and destructive diseases moved with these stocks and became widely established.

In 1914, a seed-potato certification program was started in this country. This marked the beginning of the seed-potato industry. The quality of seed potatoes has improved greatly since 1914. This improvement came about through additional knowledge gained in disease control methods by tuber indexing, tuber unit planting, and early harvesting, and the enforcement of more rigid standards by certification agencies. Recently more attention has been given to the growing of founda-

tion seed stocks. Such stocks are used for the growing of certified seed, and every precaution is taken to limit the number of diseased plants in these stocks to an irreducible minimum. The requirements for the growing of foundation seed vary slightly in different States, but, in general, all fields have to comply with the following requirements: to be eligible for certification as foundation seed, the tubers must be planted in isolated plots as tuber units and rogued early and effectively. Where aphids are apt to be abundant late in the season, the crop must also be early-harvested. The requirements for disease tolerance vary slightly in different States from not more than two percent to a fraction of one percent. Foundation seed stock must be grown under good cultural conditions, and the field must be kept free from weeds. In the field and in storage the crop must be kept under the direct control of the grower. All foundation stocks must be marketed in new bags. When they are stored in sacks, the sacks must be new. A sample of each seed lot is generally grown in an official seed-source test plot in one of the Southern States or is grown for tuber indexing in the greenhouse.

In growing certified seed potatoes, the source of the seed is important and is so considered in approving fields for certification. The regulations vary somewhat in different States, but generally the certification board insists that acceptable stock must be from foundation seed stock grown in the same State or that, if out-of-State foundation seed stock is used, it must meet the requirements of the certification board. The plots must be sufficiently isolated from potatoes of less than certified grade. The total disease tolerance is about five percent. Disease tolerance for ringrot is zero.

The first inspection is generally made as early as it is possible to identify the diseases. The second inspection is made between blossoming time and the period just before the vines mature. The field inspection is generally supplemented by a tuber inspection in storage to determine the amount of common scab, *Rhizoctonia* canker, stem-end discoloration, rots, and other diseases that cannot be detected in the field.

Continuous improvement of seed certification is doing much to improve the quality of seed potatoes and to reduce greatly the loss from diseases.

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CHEMICAL TESTS IN THE DIAGNOSIS OF PLANT VIRUS DISEASES[1]

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INTRODUCTION

Viruses occupy a unique position in the biological spectrum, for they are biological entities only when combined with a susceptible host. Purified virus preparations are claimed by the chemist. All plant viruses that have so far been purified have been found to be nucleoproteins, and are indistinguishable in gross chemical characteristics from naturally occurring plant nucleoproteins. From the chemical viewpoint, therefore, viruses are just one of a group of nucleoproteins which are found in all living organisms. However, virus particles are replicated only in susceptible hosts; therefore, from the biological viewpoint, viruses are obligate pathogens with one dimension less than 200 mμ (11). Viruses are recognized because they produce reproducible syndromes in susceptible hosts, and it is the host-virus interaction that is recognized. Some plants are symptomless carriers of certain viruses, which fact

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can be demonstrated by transmission of the virus to hosts in which symptoms are produced. It is conceivable that particles resembling viruses may exist that produce no symptoms on any known host even though they may be replicated in some hosts. Such particles would not fit the present definition of a virus unless some host was found in which symptoms were produced. Transmissibility to susceptible hosts of a particle containing nucleic acid, but smaller than bacteria, with a consequent production of a reproducible syndrome, is the distinguishing characteristic of viruses.

Virus transmission may be achieved by some form of inter-grafting of diseased and healthy plants, by vectors, or by rubbing diseased plant extracts on healthy plants. The viruses which are mechanically transmissible with plant extracts present relatively few problems in diagnosis. Hosts for these viruses are usually available which produce symptoms in a relatively short time after inoculation. Moreover, these viruses generally are replicated in high enough concentration in some host that the standard techniques for virus purification and identification can be applied. Such techniques of purification and identification include salt and isoelectric precipitation, differential and density gradient centrifugation, electrophoresis, chromatography, serology and electron microscopy. The viruses which are known to be transmitted only by vectors or by grafting present more difficult problems in diagnosis. Smith (109) describes about 300 plant virus diseases, and 56 percent of these are transmitted only by vectors or by grafting. Thus only about one-half of the known plant viruses are amenable to the usual techniques for virus purification and identification.

Difficulties in detection of viruses that are not mechanically transmissible arise particularly 1) when there is a long period between inoculation and symptom expression in the diagnostic host, 2) when different viruses induce similar syndromes on a particular host, 3) when certain varieties of a given host are symptomless carriers of a virus, and 4) when viruses occur as mixed infections. Some 45 of the several hundred known viruses are seed-borne (22), but usually the percentage transmission through the seed is low. Problems in virus detection are more common in vegetatively propagated plants, because chances are much greater for transmitting viruses from one generation to the next by propagating methods. The problem is particularly acute with fruit crops. Mechanical transmission of viruses from fruit crops is rare because virus titers are low and the tissue commonly

abounds with virus inhibitors, such as tannins. Many fruit plants carry viruses that produce few to no symptoms, so that when symptom-producing viruses occur, the plants carry mixed infections. Some viruses induce symptoms only during the first year of transmission, but the plant carries the virus throughout its life span. The problem becomes particularly difficult when such a virus is transmitted through the seed, in which case symptoms are often never produced.

A simple test for determining whether or not a plant carries a virus would be helpful in establishing virus-free understock and budwood or clonal lines of any plant. Such tests might also be helpful in control programs where destruction of virus-diseased plants is practiced, as well as in any routine procedure where it is necessary to detect viruses in large numbers of plants. Many attempts have been made to find reliable tests for such purposes with varying success. It is the purpose of this review to summarize the current information on this problem. There is one earlier review on this subject (51). The various tests are listed according to the chemical nature of the material for which the test is designed. Serological techniques might be considered a type of chemical test, but they will not be covered because of the recent review by Matthews (85). Moreover, serology, while very useful, is most applicable to mechanically transmissible viruses that occur in relatively high concentrations. Of the 32 viruses listed by Matthews (85) for which antisera have been prepared, only one is not mechanically transmissible.

PROTEIN TESTS

Cytological Stains

It has been recognized since 1903 that viruses may induce intracellular inclusions in infected cells (50), and the subject has been extensively reviewed (11, 20). The inclusions are usually proteinaceous, and some are nearly pure virus nucleoprotein (100, 111). A simple staining technique to demonstrate the presence of inclusions was devised and proposed as a diagnostic aid for virus diseases (76). The technique consisted of mounting epidermal strips or fresh sections of tissue directly in trypan blue, and observing under the microscope. In addition to crystalline inclusions, amorphous bodies were found in the cytoplasm and were designated "viroplasts" (75, 76). Better results with the method were reported when tissue was pretreated in diethyl

ether and ethyl alcohol (98). A combination stain of trypan blue with chlorazol fast pink B gave better definition of inclusions and better contrast for photography (78). The method has been used to distinguish between tulip-breaking virus (tulip virus 1) and lily latent virus (75) even though these two viruses produced similar leaf symptoms in tulip. Pea mosaic virus (*Pisum* virus 2) and bean yellow mosaic virus (*Phaseolus* virus 2) were characterized by producing isometric crystals in broad bean (77). Tobacco mosaic virus and a mild strain of tobacco etch virus were shown to occur in the same cells in mixedly infected tobacco plants by their characteristic inclusions (79). Lily rosette was readily diagnosed by the characteristic trypan blue stain of necrotic areas in the vascular elements of lily leaves (80). The only report comparing infectivity and trypan blue staining reactions concerned bean yellow mosaic virus in broad beans (99). In this case positive transmission was obtained from all leaves giving a stain test; however, transmission occurred from some leaves which did not give a staining reaction. More elaborate methods for fixing and staining tissues for virus inclusions have also been reported (9, 10, 92, 94). Characteristic staining reactions have been described for tissues infected with tobacco mosaic, potato X, squash mosaic, spotted wilt, potato leafroll, beet curly top, and Pierce's disease of grapes.

It is difficult to evaluate the usefulness of these staining techniques because they have not been applied to many of the virus diseases that are difficult to diagnose and because various sources of possible confusing reactions have not been studied. The trypan blue method has the advantage of simplicity but the more elaborate techniques have the advantage of fluidity, in that they can be varied for different viruses and different hosts. Though it is clear that many viruses do not induce the formation of inclusion bodies, and that inclusions may not be formed if virus titer is low (99), these methods appear to be useful diagnostic aids for certain viruses, and warrant further study of their application.

General Protein Tests

Since all viruses that have been purified are known to be nucleoproteins it is natural that general protein tests would be tried for diagnostic purposes. The "Biuret Test" was first proposed for the diagnosis of potato viruses (27). In this test an alkaline solution of copper sulfate gives a violet color with proteins. When expressed

juice from diseased tubers was tested it gave a violet color, whereas juice from healthy tubers yielded a yellow color. A later report (81) indicated that this test was not reliable. In Japan the "Biuret Test" has been proposed for different virus diseases (35–41, 43) although a critical evaluation of the method seems not to have been undertaken. Some of the color reactions obtained may also have been due to polyphenols rather than proteins. When potatoes were studied with the quantitative methods of chromatography and paper electrophoresis, it was found that tubers infected with potato leafroll actually had a lower protein content (73).

For viruses that occur in relatively high concentration in infected plants, diagnostic tests based on protein precipitation reactions have been suggested. Thus tobacco mosaic virus and potato virus X could be recognized because a 10 percent sodium chloride extract of infected leaves remained turbid for days whereas extracts of healthy tissue cleared on standing overnight (52). Juice from frozen plants reacted similarly when 10 percent acetic acid was added. Extracts from diseased plants that were treated with alcohol or mercuric chloride gave more obvious precipitates than similar extracts from healthy plants (45).

The general protein tests do not appear to be of much value as diagnostic aids for virus diseases. For viruses that occur in high concentration, other means of diagnosis are much more reliable than precipitation tests of plant extracts. Moreover, a number of conditions other than virus diseases affect the protein level of tissue.

AMIDE AND AMINO ACID TESTS

Amide and amino acid tests have been used principally for potato leafroll. Chromatographic work has indicated that leafroll tubers are low in free tryptophane and tyrosine (5), but a test was proposed based on a higher total tryptophane content of diseased tissue (103). These reports have not been confirmed. Other work on potato leafroll (1) has indicated that glutamine is two to three times higher than normal in diseased tubers. A simple color test for leafroll with Nessler's reagent was based on the higher glutamine content (21), but later work (34) indicated that the method was not consistent. A test for the rough-skin disease of apple trees based on a higher arginine content of the tissue has also been reported (19). No consistent evidence indicates that amide or amino acid tests are reliable diagnostic

aids for virus diseases. It would be expected that various environmental conditions might affect the level of the commonly occurring amides and amino acids so that tests based on them would not be too reliable.

ENZYME TESTS

Several tests for viruses based on differences between healthy and diseased tissue in enzymes associated with electron transfer have been proposed. One of the first such tests involved the insertion of a sheet of copper in a potato tuber followed by holding the tuber eight hours at 37° C and 16 hours at room temperature (8, 14, 15). Healthy tubers reacted by yielding a black melanin pigment around the copper probe, while diseased tubers yielded little to no melanin. The method was considered to be of limited diagnostic value because of variability between varieties and within varieties from different localities (61).

Another method for testing diseased potato tubers involved the rate of fading of an iodine stain of cut tubers (44, 118). Diseased tubers caused the iodine-starch stain to fade more rapidly than healthy tubers. The accuracy of the method was reported to be 70 percent immediately after harvest, but was much lower after sprouting of the tubers. The time of reduction of methylene blue was the basis for another test (106, 119). Extracts of diseased tissue reduced methylene blue faster than extracts of healthy tissue. The method was tested on virus diseases of potatoes, radishes, turnips, and broad beans and was reported to be 70 per cent effective (46).

An apparently more reliable method consisted of using a solution of 2-6 dichlorophenol-indophenol decolorized with sodium bisulfite (82). Sap from diseased material turned the reagent blue while healthy sap did not. Dahlia mosaic infection was detected in symptomless dahlia plants with a high degree of accuracy using this technique. Tobacco mosaic virus and potato virus Y infection were reported to be detected in tobacco within 42 hours after inoculation. This test was later tried on sap expressed from young potato shoots (84), and was reported to give 95 percent agreement with serological tests on 1000 tubers. Differences from serological tests with this method were mainly due to apparently healthy tubers which gave a positive color test. 2,3,5-Triphenyl tetrazolium chloride has also been used as an indicator for virus infection (13). Virus diseased tissue reduces the dye faster than healthy tissue. Southern bean mosaic, common bean mosaic, and pod mottle were de-

tected in beans; beet yellows and curly top in sugar beets; cucumber mosaic in sweet corn and tobacco; tobacco mosaic in tobacco; and chrysanthemum stunt in chrysanthemum.

A more quantitative method has been proposed for ratoon stunting disease of sugar cane (7). The colored formazan is extracted with hot acetone and estimated in a colorimeter. The test is not specific for viruses, because bean tissue treated with 2,4-dichlorophenoxy-acetic acid is known to produce a similar color reaction with the reagent (28).

CARBOHYDRATE TESTS

The observation that starch metabolism in the leaf is affected by virus infection was first noted by Holmes in 1931 (48), and is the basis for quantitative assay of viruses by local starch lesions. Starch is synthesized in light at a slower rate in infected tissue, but also is not hydrolyzed as rapidly in the dark. Leaves to be tested are cleared of chlorophyll by heating with alcohol, and the starch stained with iodine-potassium iodide solution. The method is of some diagnostic value since in a given host the type of starch lesion produced is characteristic for a given virus; however, the method is limited to recently mechanically inoculated leaves.

In the tristeza disease of citrus, starch accumulates in parenchyma above the bud union of sweet orange grafted on sour orange stock. A 3 per cent alcoholic solution of iodine applied to the superficially scraped bark at the bud union has been used as a diagnostic aid for this disease (17).

A test for potato leafroll was based on the accumulation of glucose in mature potato leaves infected with the leafroll virus (83). A drop of centrifuged sap was placed on filter paper and air-dried. A saturated solution of aniline phthalate was then atomized on the paper, and spots from leafroll-infected tissue turned blackish brown, while those from healthy tissue were faint yellow. The reliability of this method has not been ascertained.

Another test for potato leafroll was based on the observation that callose formation was much more marked in diseased plants (110). The callose was stained with corallin soda or resorcin and observed microscopically. One thousand five hundred twenty tubers were tested with an accuracy of 71 per cent. The test was not considered reliable enough for diagnosis but may be an aid for other types of diagnosis. More

recent work has confirmed these results (88, 107, 112). One worker reported 96 per cent agreement between the callose test and field indexing (104). The callose test has also been successful in detecting spindle sprout of potatoes and was reported to be more reliable for spindle sprout than leafroll (120).

Several diagnostic tests based on a color reaction with phloroglucinol and HCl or with HCl alone have been used. These tests are grouped together because they seem to owe their reaction to the presence of pentoses (or condensed pentoses). The color reaction is usually confined to certain cells or tissues in infected plants. While it is recognized that those plant viruses that have been purified contain ribose, the color reactions referred to here are very unlikely due to the virus itself, but rather seem to be a manifestation of a disturbed metabolism in the host wherein pentoses, or condensed pentoses, accumulate in certain cells.

It has been known since 1913 that phloem degeneration in potato plants affected with potato leafroll is characterized by a red stain with phloroglucinol-HCl (91). The earlier work has been summarized in other reviews (20, 24). The method has been used as a valuable diagnostic aid for potato leafroll in various countries (62, 105, 121).

The technique consists of immersing a 0.5-mm section of potato stem in a 1 per cent alcoholic phloroglucinol solution for one minute, followed by concentrated HCl until the xylem stains red. The sections are rinsed and examined at 40× magnification. In healthy tissue the xylem and fibers stain red, while in leafroll affected tissue both the inner and outer phloem also show a red stain in certain areas. The phloroglucinol-HCl test has also been applied to exocortis infection in *Poncirus trifoliata* L. where certain ray cells of the bark stain in infected tissue (18). Of 215 diseased specimens tested, 98.6 per cent gave a positive color test.

A test that appears to be related to the above is the acid test for phony disease of peach (49). This test appears to be specific for phony peach. In this test acidulated methyl alcohol is applied to sections of the wood from peach roots, and a positive reaction is indicated by certain cells staining red to purple. It is likely that phloroglucinol or other aldehyde-coupling phenols are either already present in the peach tissue or are released by the acidulated alcohol, so that the reactions involved are similar to the phloroglucinol-HCl test. In fact, if methanol is satu-

rated with zinc chloride and 2 per cent phloroglucinol and 20 per cent HCl added at 10:1:1, a test that approximates the acidulated methanol test is given with peach tissue, except it is more sensitive and less specific (57). Peach rosette infected tissue also gives a test with this procedure but does not with the acidulated methanol test. An anatomical study of phony peach diseased roots (25) has shown that the staining results from plugged vessels and thickened walls of neighboring cells. The acid test for phony peach has been a very useful diagnostic aid for this disease (49, 55, 56, 58), and was one of the first chemical tests to be used extensively as a diagnostic aid.

Acidulated methanol was also used as a test for swollen shoot disease of cacao (31), where diseased wood sections assumed a magenta color faster than healthy sections. However, the test was not positive enough to be of wide use.

A modified phloroglucinol test was suggested for the detection of peach mosaic in peach (108). In this test free-hand sections of roots or stems were placed in a watch glass and a drop of saturated phloroglucinol in methanol added. After this had evaporated to dryness a drop of nitrophenolic acid in methanol was placed on each section and again allowed to evaporate to dryness. The xylem of sections from trees affected with peach mosaic turned pink to lilac, while sections from healthy trees were yellow. The test was applicable only on dormant wood, and has not been confirmed.

A recent study on the leafroll diseases of grapevines has shown that cellulose and lignin decompose in infected plants through the intermediate compound 4-methyl-d-glucuronic acid (87). This compound was detected by paper chromatography in 625 leafroll infected plants but not in 700 healthy plants or in plants infected with other diseases.

POLYPHENOL TESTS

The term "polyphenol" perhaps needs a little clarification. Basically any phenol with two or more hydroxyl groups is a polyphenol, and the term is used here to include not only the simple but the conjugated polyphenols, and includes such materials as tannins, flavones and anthocyanins.

Resühr (97) was one of the first to recognize that virus infection induces a higher polyphenol content in plants. He induced many virus-like symptoms in various plants by injecting various tannins into them,

and it was his opinion that many of the symptoms caused by viruses are due to the accumulation of tannins in the plant. Other workers have also reported the accumulation of polyphenols in diseased tissue. Peach leaves affected with buckskin disease were shown to accumulate phenolic materials (93), and cotton, tomato, tobacco and lilac plants affected with "yellows-type" viruses were reported to accumulate tannins (53). The specific polyphenol, scopoletin, accumulated in tobacco plants affected with tomato spotted wilt (16), and in potato plants affected with leafroll and other viruses (3, 96).

The introduction of simple tests for virus diseases of woody plants, based on the accumulation of polyphenols in diseased tissue (65, 66, 70, 71), stimulated considerable interest because virus diseases of woody plants are often difficult to diagnose by conventional methods. The tests were based on the production of a red color by diseased tissue when leaf discs were heated in sodium hydroxide and the resultant extract oxidized. Oxidation was achieved with a trace of cupric ion in the sodium hydroxide (65) or by shaking the extract in air (70). The latter procedure was more suitable for tissue with high sugar concentration such as cherry fruit tissue.

Another procedure consisted of clearing leaf discs first in alcohol, then heating with an acidulated alcohol to give a red color (67). If chlorophyll was removed from whole leaves by heating in alcohol containing formaldehyde, and the leaf then heated in sodium hydroxide, the polyphenols remained fixed in the leaf and gave a blue color. The distribution of the blue color was characteristic for certain types of diseases and in general followed the pattern of symptom expression (66).

Since the accumulation of polyphenols, in general, seems to coincide with the distribution of viruses in the tissue, one might speculate that polyphenols may be a defense mechanism of the plant against virus infection. A number of workers have shown that certain polyphenols are virus precipitants (12, 114, 115, 125), and that plant extracts high in polyphenols inhibit virus infection. It is thus not unreasonable to suppose that polyphenols accumulate because they are virus precipitants.

The accumulation of polyphenols is not peculiar to virus infections. Other conditions, such as girdling from any cause, may induce false readings (71). Certain types of low-temperature injury, heavy San Jose scale infestations, *Coryneum* blight infections, and red spots on peach leaves from arsenic injury or nitrogen deficiency may give mis-

leading results and should be avoided in sampling. Thus, careful selection of samples is a prime requisite in these tests.

The materials that accumulate in virus-diseased tissue of fruit trees which are responsible for these color reactions have not been specifically identified. One report in the literature (54) mistakenly assumed that the color test with alkaline copper solution is due to reducing substances. Though it is true that high concentrations of reducing sugars will induce a precipitate of copper oxide with the reagent, no color is imparted to the solution. Moreover, the red color can be obtained without the use of copper (70) so that reducing sugars play no part in the reaction. A chromatographic study of flavonoid constituents of cherry leaves affected with rusty mottle and peach leaves affected with Western X disease showed no qualitative differences; however, the virus-affected leaves contained more of all constituents (eight or more) and much more of some (29). Diseased cherry leaves contained three times as much kampferol and quercetin and nearly twice as much caffeic acid as normal leaves. Diseased peach leaves were particularly high in chlorogenic acid and an unidentified component. A recent report (64) concerns the detection of pear mosaic and peach ringspot by paper chromatography. In cacao, the material responsible for a red color when leaf extracts are heated with alkali has been reported to be a leuco-anthocyanin (47). Scopoletin is also known to give a red color when heated with alkali in the presence of a trace of copper (4). These color reactions are thus not specific for any particular substance, but may be given by a number of different polyphenols.

The polyphenol tests have been applied to different kinds of plants with variable results. The staining test applied to strawberries (86) was reported to work well on severely infected plants but not on mildly affected ones, and it was thought the method might have value in diagnosing the relative amounts of virus infection in a given lot of plants. In more recent work (23) chlorogenic acid was detected by paper chromatography in crinkle- and yellows-infected strawberry plants but not in plants infected with Latent A or Frazier's mild mottle viruses. Both the color test and the staining test showed considerable promise as a diagnostic aid for elm phloem necrosis (2). A modification of the color test was used for detecting swollen shoot disease of cacao (116), but a later report thought the method was not reliable enough for wide application because other factors than virus infection may induce the accumulation of polyphenols (47). The color test was used to detect

infectious degeneration of grapes (90), but another report on detection of grape virus diseases with this test (30) indicated that the results were variable, partly due to sampling difficulties, and it was also assumed that no viruses would be transmitted through grape seeds, which has not been proven.

The application of the methods to various stone fruit viruses have also been variable. One report indicated the staining method most suitable, although none of the methods was satisfactory for plum pox (72). Varying results were also reported when the color test was applied to virus diseases of almonds, plums, figs, peaches, and apples (101). The variable results obtained suggest that these tests are not specific for virus diseases and particular attention must be given to choosing adequate samples free of confusing disorders, a precaution true of any chemical test for plant virus diseases. This precaution, of course, limits the usefulness of the method.

Another factor that is sometimes overlooked is the presence of viruses in hosts which appear healthy. This may be illustrated by Kwanzan and Shiro-fugen flowering cherries which always give very strong polyphenol tests, and for which it was predicted that they must be carrying a virus or viruses that produced no symptoms (69). Later it was shown that these cherry varieties almost universally carry a virus that produces a disease of the little cherry type in sweet cherries (95). Thus, care must be taken not to assume that a plant is virus-free merely because no symptoms are evident.

The polyphenol tests have been helpful in the search for vectors of fruit tree virus diseases (6, 122, 123, 124). Inoculum sources were tested and vector suspects allowed to feed on those sources that had the highest virus titer. In addition, large numbers of test plants were screened for possible virus transmission before obvious symptom expression occurred.

NUCLEIC ACID TESTS

A procedure has been proposed for detecting virus diseases in herbaceous plants as well as in fruit trees by determining the ultraviolet absorption spectra of leaf extracts (67). Chlorophyll was removed from discs of leaf tissue by heating in alcohol, and the discs then extracted with acidulated alcohol. Characteristic absorption curves for certain diseases were obtained. Extracts from tissue affected with mosaic type diseases gave higher absorption at 260 mμ, presumably owing to

a higher nucleic acid content, while extracts from tissue affected with yellows type diseases gave higher absorption at 280 mμ, presumably owing to a higher polyphenol content. The technique has been adapted to the quantitative assay of stone-fruit ring-spot virus in cucumber (59) and has been very useful for chemotherapy studies of this virus (60, 68) because no other methods for assaying this very unstable virus were available. It has also been used for a quick assay for tobacco mosaic virus in tomato plants (60).

The method has been very helpful in detecting Western X disease of peach in a search for vectors of this disease (6, 122, 123, 124). Several virus diseases of tobacco have been studied with this procedure (89), and tobacco mosaic and potato X could be detected, but the method was not satisfactory for potato Y or bouquet disease. Two reports on the unsuccessful application of the method (63, 117) probably result from insufficient care in choice of samples. The procedure requires rigid control of samples of healthy material.

Paper electrophoresis of a preparation from tulip bulbs has recently been reported to show two nucleic acid fractions in healthy bulbs but three in diseased (42). The method has not been confirmed.

UNCLASSIFIED TESTS

The dye, acridine orange, has been used as a test for leafroll of potatoes (33). In this test the dye (.005 per cent acridine orange, .01 per cent Bordeaux red, 0.3M KCl at pH 3.7–4.0) was applied to tuber slices and exposed to light for 15 minutes. Leafroll tubers gave a visible red stain. This test has not been confirmed. Another unconfirmed test concerns the ratoon stunting disease of sugar cane (26). Sections were cut from the periphery of mature basal nodes of cane, 3 per cent hydrogen peroxide applied for 10–15 seconds, then blotted off and concentrated HCl added. A blue-green color developed at the leaf-scar level around vascular bundles in healthy cane, but little to no color developed in diseased canes.

Fluorescence of cut potato tubers in ultraviolet light was a technique used to detect potato virus diseases (74). Over 1000 tubers were examined and those with low fluorescence produced 31 per cent healthy plants while those with a high fluorescence produced no healthy plants. The location and color of the fluorescence was in some degree characteristic for given virus diseases. A further study of this procedure (32)

could not confirm these results. Although the removal of all fluorescing tubers would greatly reduce the percentage of virus infection in a given seed lot, there would still be some diseased tubers left.

Fluorescence of an extract of carnation plants appears to be a valuable diagnostic aid for carnation mosaic (113). The extract was prepared by autoclaving a portion of the vegetative shoot in distilled water for 45 minutes at 15 pounds pressure. The extract was shaken with butanol and a drop of ammonium hydroxide added. When examined under ultraviolet light, extracts of mosaic-infected plants produced a light pink color at the interface between the water and the butanol. No fluorescence was observed in extracts from healthy plants. The reliability of the test was determined by comparing results obtained from inoculation to a diagnostic host. Over a three-year period, 35,763 plants were tested in this way. By the ultraviolet test, 35,670 were found to be infected, while 35,678 were found to be infected by the inoculation test. While it is not known what conditions might interfere with this ultraviolet test, the method would appear to be reliable.

SUMMARY

Viruses are recognized because they produce characteristic syndromes in susceptible hosts. The biological test of transmissibility to susceptible hosts is therefore the standard diagnostic test for viruses and is the most sensitive test known. As little as 5×10^{-16} gm. of tobacco mosaic virus can be detected in tobacco by transmission tests (102). Transmission tests, however, are sometimes tedious and time-consuming, and this is particularly true of those viruses transmitted only by insect vectors or by grafting.

We often need a rapid diagnostic test, especially where large numbers of plants are to be studied. An ideal chemical test would be both rapid and specific for viruses alone, but no such tests are known. The difficulty with specificity is that, chemically, viruses are very similar to naturally occurring host nucleoproteins, and differences occur only because of the sequential arrangement of the nucleotides on the nucleic acid chain. There are no known methods available to detect this difference in arrangement except biological tests.

Serological tests, next in order of specificity, are very useful for those viruses that are mechanically transmissible. Although the relatively few viruses that have been purified have been shown to be nucleoproteins,

there is no reason to suppose that all viruses contain protein, and some may be pure nucleic acid. Nucleic acid is not antigenic and serological tests would be applicable only to those viruses that induced the formation of a specific antigenic protein in the host.

Most of the various chemical tests proposed for virus diseases are based on the accumulation of products in the host resulting from the virus-host interaction. Although the lack of specificity of these tests limits their applicability, the view that they are of no value would seem to be as extreme as the view that the tests may be used indiscriminately. Some of the tests have been shown to have definite diagnostic value under certain conditions when reasonable care and judgment are used in sampling. Such tests would include (a) protein-staining tests for certain bulb and legume virus diseases (75, 80); (b) the color test for oxidizing enzymes for dahlia mosaic and possibly certain potato virus diseases (82, 84); (c) the phloroglucinol test (pentoses) for potato leafroll (62, 105, 121) and for exocortis in *Poncirus* (18) and the related acidulated methanol test for phony disease of peach (49, 55, 56, 58); (d) the callose test for leafroll and spindle sprout of potatoes (88, 107, 110, 112, 120); (e) the 4-methyl-d-glucuronic acid test for leafroll of grapevines (87); (f) the polyphenol tests for certain stone fruit virus diseases (65, 71), and for swollen shoot of cacao (47, 116); (g) the ultraviolet test for carnation mosaic (113); and (h) the nucleic acid test for stone fruit ring spot in cucumber (59, 60, 67, 68).

Chemical tests for virus diseases would thus seem to have a definite place as a diagnostic aid in the virologist's armamentarium.

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TECHNIQUES FOR THE OBSERVATION AND ISOLATION OF SOIL MICROORGANISMS[1]

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INTRODUCTION

The soil serves as a reservoir for many microbial pathogens of plants and animals, antibiotic-producing, and carbon- and nitrogen-cycle microorganisms, as well as myriads of other lower plants and animals which play a part in the soil economy. The ability to detect and if possible to isolate these organisms in pure or mixed culture has become of increasing importance to soil microbiologists. The techniques available for doing this are necessarily selective. While this fact should work to our advantage when we are interested in particular taxa already characterized, it is a disadvantage when dealing with unknown entities or when making broad qualitative studies. With the current interest and concomitantly increasing activity in soil microbiological problems, the available techniques, originally borrowed from medical bacteriology, have been greatly extended. Yet, it is evident that we are still able to obtain *in vitro* only a portion of the total soil microorganism population.

Some of this difficulty is, perhaps, more apparent than real and stems from the difficulty with which information is interchanged among the diverse fields now active in studying soil microbiological problems. Those in one field may be unaware of techniques which have been applied elsewhere to similar problems. In other instances workers "discovering" a technique may have only duplicated one which was in use for some time in another field.

However, two very real difficulties connected with the isolation of soil microorganisms are: (a) the inability of some organisms to grow and complete their life cycle in a defined synthetic medium, and (b) contamination, especially by bacteria and fast-growing fungi. Until such time as these problems can be overcome, rigorous experimentation in some areas will be greatly hampered. The advent of antibiotics has largely eliminated the problem of contamination; on the other hand, much remains to be done with regard to chemically defining culture media. The creation of favorable physical conditions, *e.g.*, temperature, light, and osmotic concentration, incorporation of suitable carbon, energy, and nitrogen sources, as well as micro-nutrients and "growth factors," and provision for removal of possible inhibiting metabolic waste products are among the problems which must be considered. In addition, certain factors in the environment may have to be altered during the life cycle. Further advance depends upon our ability to control more

stringently the isolation environment, upon our ingenuity, and upon observation and knowledge of microbial physiology.

There is no single universal technique. Combinations may be used with greater benefit; each additional technique employed, however, further limits the spectrum of organisms which can be obtained. Thus, experimental results obtained and interpretations made depend to a large extent upon the isolation techniques used to derive the results. It is, therefore, of great importance that the investigator be cognizant of the limitations of the techniques employed.

In this article the term soil is used in its most general sense, including, besides the soil proper, all organic matter which might be found in it, as well as natural waters. The groups to be discussed include algae, bacteria, fungi, nematodes, and protozoa. The techniques for their isolation are arranged for the sake of convenience of presentation under three main headings: physical, chemical, and biological.

Along with these techniques are briefly discussed the various methods available for observing soil organisms as they occur *in situ* in the soil. Sometimes, examination as a prelude to isolation may yield valuable additional information, as well as suggest to the investigator possible isolation techniques. Techniques for isolating fungi (169, 359), algae (31, 204, 261, 282), nematodes (133, 278, 370), and invertebrate metazoa (94) have been previously reviewed.

ISOLATION TECHNIQUES

Physical Methods

MIGRATION. Advantage has been taken of the fact that many organisms have a motile stage that exhibits some type of tactic movement. By setting up the appropriate environmental conditions, organisms can be made to migrate relatively far through a sterile liquid (65) or semi-solid medium (328), or over the surface of a solid one. As they move toward the more favorable region they rid themselves of most contaminants, literally washing themselves clean as they move.

Ogata (250) prepared glass columns containing sterile water into the bottom of which negatively geotactic protozoa or algae were introduced. Later sterile organisms could be drawn off at the top. Others have successfully applied the technique to algae (31, 261), protozoa (177, 213, 263) or dino-flagellates (148). This last group may move, due to a tactic response, as much as 10 meters in 12 hours. A reversal of

this procedure may afford separation of protozoa which appear to be positively geotactic (177). Ogata's basic apparatus was improved upon by Glaser and Coria (128, 129). Later they further modified the technique by first centrifuging and washing the suspension of organisms three times before passing them through columns (130). However, the results of Hetherington's experiments suggest that they still probably did not obtain completely sterile protozoa (157). He found that if the time allowed for migration is relatively short, the protozoa may still retain bacterial spores ingested prior to the treatment. This fault could largely be overcome by allowing the organisms to remain in sterile water for several hours after the migration process and then passing them through a column for a second time (156).

Zoospores of *Rhizophlyctis rosea* are strongly aerotactic and accumulate at the surface of a liquid culture (307). These provide an abundant source of inoculum for culturing with a minimum of contamination.

Zoospores of some algae and Phycomycetes are phototactic. A variety of isolation techniques have been employed, based upon this fact, especially for marine Phaeophyceae and Chlorophyceae (25, 31, 204). In some instances there appears to be a correspondence between the photosynthetic action spectrum and the phototactic action spectrum within groups of microorganisms. *Euglena* spp. move toward the blue-green portion, while the purple bacteria congregate in the infra-red and in lesser bands in the yellow and green regions (25, 331). Paramphistome metacercariae are attracted to iluminated yellow surfaces (100).

Organisms which are normally migratory may be decontaminated by much the same method as above. Oehler obtained pure cultures of protozoan flagellates and ciliates by allowing them to migrate over an agar surface (248). The plasmodia of Myxomycetes have been allowed to migrate several times over a non-nutrient agar, only a small portion from the advancing tip being transferred to new agar each time (69). Also, sorocarps which are formed from portions of the plasmodium which have migrated outside the zone of bacterial colonization usually contain sterile spores (266). One of the oldest methods for obtaining monoxenic amoebae cultures was presented by Beijerinck (24). Plates were streaked radially with a known bacteria and the contaminated amoebae introduced at the center. After migrating through the bacterial colony to the edge of the dish the amoebae are usually free from all

but the streaked bacteria. The same general technique has been used with Myxobacteria (292) and Myxomycetes (181).

In 1905 Berlese (27, 369) published on a method, which bears his name, for the collection of small arthropods from soil litter. He utilized a double-walled funnel, the jacket of which contained water heated to 105–110°F. The arthropods in the litter, which was placed in the inner funnel, were stimulated to move downward away from the light and toward the warmth. Upon reaching the neck of the funnel, they would fall into a collection bottle which had been placed under the funnel.

Tullgren (341), and later Tragardh (338) and Haarløv (137), improved upon Berlese's technique by continuously illuminating the funnel from above and enclosing it in a box. This has the advantage not only of maintaining constant light, but also slowly dries out the contents of the funnel from the top downward, thus creating an additional stimulus for the downward movement of the arthropods. Temperature and moisture gradients are further magnified in the multiple Tullgren apparatus of Macfadyen (219).

An apparatus somewhat parallel in principle, the Baermann funnel (15), is now widely used for the collection of nematodes. Here, the sample to be extracted is placed in a porous container, usually made of cloth, filter paper (206), or cleansing tissue (232), which is suspended in a glass funnel partially filled with water, so that the sample is not completely immersed in the water. The nematodes move into the water and fall to the bottom of the neck of the funnel which is closed, usually by a rubber tube and pinch clamp or, alternately, a capillary tube (308).

Numerous modifications of this technique have been reported. Their main attributes are cheap unit cost and/or simplicity, and as a corollary the operator can usually survey a larger number of soil samples in a shorter time. Some of these modifications, as well as other useful nematode-isolating techniques, have been recently reviewed by Cairns and Oostenbrink (278). Expendable paper cups have been used in place of the glass funnels (8, 108, 232). Using a tube with a screen over the end, to hold the cleansing tissue and soil sample, is reported to increase the cleanliness of the nematode suspension obtained (355). Substitution of thin polyethylene tubing for rubber tubing will allow continual observation of the contents and permit exchange of gases without water loss. In this case the nematodes remain active much

longer (312). When samples are taken from the Baermann funnel and allowed to stand for 15 to 20 minutes, the large majority of the nemas are found on the bottom of the vessel; they can then be drawn off with a pipette and examined microscopically (108). Samples may be taken directly from the Baermann funnel and passed through a Büchner funnel, the nematodes remaining on the surface of the fritted glass from which they are removed by flushing with a small volume of water (112). A more rapid method employs a Millipore filter attached to the end of a syringe in place of the Büchner funnel (313). Higher yields of nematodes, rotifers, and tardigrades have been obtained when the Baermann funnel apparatus is enclosed in a lighted box (253).

The Enchytraeid worms, because of their low resistance to desiccation, are not efficiently extracted by the foregoing method. Overgaard Nielsen (254) has devised an apparatus for extracting them from soil which slowly, over a period of several hours, sets up a temperature and moisture gradient within a cylinder containing the soil sample. The organisms move upward through the sample and into quartz sand, which has been placed over the sample. The sand is removed from the cylinder and the Enchytraeidae separated by flotation.

Migratory endo-parasitic nematodes have been recovered from plant roots by incubating the roots in polyethylene bags (323) or in moist chambers for several days. With the latter method, the roots are syringed periodically and the washings examined for the nemas that have migrated into the water phase (376). According to West (364), samples incubated while completely immersed in water for several days give greater nematode recoveries. By using this modification only a single examination of the sample is necessary.

MACERATION. Slow-growing fungi can be isolated from plant tissues by comminuting the tissues in a Waring blendor, then diluting and plating out the suspension. Although this method results in higher colony counts, it effectively isolates plant portions containing only the slow-growing organisms (314, 315, 316). This treatment may also be employed as the initial step in isolating nematodes from within roots (48, 90, 115, 326). Pectic-enzyme preparations have also been used to macerate roots infected by nematodes (97).

Warcup has isolated a wide range of fungi from infected roots by placing a small piece of root in a Petri dish, then tearing it into fragments with needles and dispersing the fragments in agar (360).

FLOTATION. Some structures such as spores or larvae will float when

water is mixed with soil. The majority of the organisms are, however, found in the sediment with the soil. If a solution of a specific gravity higher than that of water such as sucrose (48), sodium silicate (365), sodium thiosulphate (317), brine (1, 104), or magnesium sulfate (9, 190) is used, many of the organisms are now found in the supernatant fraction while the soil is still retained in the sediment. Magnesium sulfate also has the ability to flocculate the soil colloids, thus further reducing the amount of mineral matter remaining in suspension (190). Passing a stream of fine air bubbles through the suspension helps to free insects from the soil particles (190). Organisms can be further isolated by subsequently screening (83) or centrifuging (111, 203, 310) the supernatant portion. The addition of a small amount of mineral oil to an aqueous soil suspension results in 80 to 90 per cent of the spores of *Helminthosporium sativum* collecting in the upper emulsion layer (198, 199).

Soil arthropods can be separated from other material—mostly vegetative—by adding benzene or xylene to the mixture and shaking. The arthropods are found in the organic layer while the vegetation remains in the water phase. The organic layer can be easily separated out by cooling to 5°C, at which temperature the benzene freezes; the solid plug is then removed, thawed, and the benzene drawn off by filtration, leaving the organisms (267).

SEDIMENTATION. By virtue of their density, larvae, cysts, eggs, mature nematodes, and spores can be collected and/or washed by means of centrifugation. Repeated low-speed cycling with sterile water has in itself largely freed organisms of contaminants (113, 178, 293). A density gradient can be established within a single centrifuge tube by layering sucrose solutions of varying density in it (34, 35). Spores, which have a density intermediate between soil and plant material, have been collected by this method (251). Caveness and Jensen (48) used a method for separating nematodes that consists of centrifuging the material first in water, then recentrifuging the centrifugate in an aqueous sugar solution. This second supernatant portion is then quickly diluted with water and the nematodes allowed to settle. A modification involves pouring immediately the sugar-water supernatant through a screen which leaves the nematodes on the screen. They are then rinsed and washed from the screen for examination (233).

Because the sedimentation rate of the various components in a heterogeneous suspension varies, the bottom deposit can be taken at various

time intervals to give a partial separation of the components based primarily on their density, shape, and charge. Essentially all the bacteria are associated with the colloidal matter which does not settle for five hours. By contrast, *Pythium* spp. are found associated with the coarser soil fractions (10.) Nematodes have also been collected by sedimentation (284). According to Chapman (51), this method gives better results than either the Baermann funnel technique alone or when combined with sieving.

ELUTRIATION. Another method which takes advantage of differential settling velocities is elutriation. Most of the current apparatus are based in principle on the single-tube elutriator developed in 1867 by Schöne for soil studies (281). It consisted of a vertical tube through which water flowed from bottom to top with a given velocity. The flow rate was adjusted so that the particles, lighter ones first, slowly rose to the top where they could be collected. Application of this method by Seinhorst and others to nematode collection has resulted in a high percentage of recovery from clay soils (68, 171, 370). A device which combines this method with a subsequent series of sieves, thus allowing one to trap different sized nematodes selectively, has been devised (324) and modified (221). Another device which first employs a sieve and then applies the principle of elutriation has been used to collect nematode cysts (190).

DESICCATION. Resistant structures such as spores, cysts, and sclerotia can tolerate desiccation whereas actively proliferating organisms usually cannot. Tsujitani in 1896 obtained sterile protozoa by placing their cysts for several weeks in a desiccator over acid (339). Drying soil samples for several days over calcium chloride has been reported to kill mycelia but not spores (218). Even the mature oogonia of some Phycomycetes have remained viable when air dried for several years (70). Species of algae exhibit a differential resistance to drying and in certain instances may be separated one from the other because of this attribute (167, 282). It is doubtful, however, if such a treatment in this case would eliminate contamination.

CONCENTRATION. High concentrations of carbon dioxide may selectively inhibit the growth rate of certain organisms while allowing others to grow normally (99, 189). When dilution plates are incubated in environments containing high concentrations of carbon dioxide, some fungi are found which do not develop when the plates are incubated in a normal atmosphere (29). Certain fungi causing storage

deterioration of grain often do not grow on media of low osmotic concentration and require media containing 15 to 20 per cent sodium chloride or 40 to 80 per cent sucrose (59). It would be worthwhile to attempt to isolate soil microorganisms on such media. It is known that many organisms occurring in bodies of water having a high salt concentration will initially grow only on media containing high salt concentrations. On the other hand, some of the aquatic Phycomycetes are unable to survive on media having relatively high osmotic concentrations (102).

COLORATION. Fults et al. (120) found that parasitic races of *Streptomyces scabies* plated on agar containing 2, 3, 5-triphenyltetrazolium chloride produced a significantly greater color intensity than did nonparasitic races. It has been suggested that this compound might also be used to detect the presence of enteric pathogens (197) and pathogenic clones of *Pseudomonas solanacearum* (175). Other workers have shown that this chemical may facilitate the differentiation of species or variants within a single species (279); however, a number of different microorganisms can react with it (270). Two other media making possible color differentiations have been reported. In a medium containing tyrosine (227), virulent clones of *S. scabies* produced a melanin-like pigment. Microsclerotia-producing clones of *Verticillium albo-atrum,* but not most other fungi, appear black when grown on an ethanol agar medium (244).

WASHING. A large majority, if not all, of the surface contaminants can sometimes be eliminated by washing the organisms in water, either in a flowing system (53, 291) or by transferring them serially through small volumes of sterile water. Hargitt and Fray (142) were able to obtain pure cultures of *Paramecium* spp. by passing them through five serial washes. Later, Parpart (255) increased the number of washes to nine and allowed the protozoans to remain in the fifth wash for five hours. This modification enabled him to eliminate bacterial spores which might have been ingested before the treatment was begun. Larger protozoa are more difficult to free from contaminants by this method (156).

The wash water may be checked by plating it out to determine the completeness of removal of fungus spores and bacteria (143). The use of complex media, instead of water, as a washing medium has also been advocated (156). Besides protozoa (212), this technique has been

used with algae (61, 282), nematodes (378), and in the study of mycelia on and within roots (143, 147, 348).

DILUTION. One of the earliest, and still most widely used, techniques is the dilution plate (351). In essence, this method attempts to separate wanted individuals spatially one from another and, as a corollary, to separate these individuals from contaminants. It has been described in detail by Johnson et al. (169). Another approach to this technique is a mechanical continuous-flow apparatus which logarithmically dilutes the soil suspension (226) and appears to eliminate not only some of the sources of human and mechanical error, but also should minimize biological variation due to inhibitory effects between colonies.

SIEVING. Screening soil or plant material isolates relatively large structures such as sclerotia (179, 195), arthropods (237), and nematodes (60, 67, 133, 285). Mechanical apparatuses for passing material through a series of size-graded screens with running water have been devised (237, 276). With modification, the material on each screen can be collected continuously (290). A frequently used technique for removing nematodes from the soil first involves screening the soil into several size fractions, then placing each fraction in a Baermann funnel apparatus (60). Nematodes may also be transported from one screen to another by vacuum rather than by gravity or running water (140). If the contents of each sieve are placed in a vessel with water, small organisms can be collected by flotation (190).

Molecular filter membranes are being used to remove quantitatively the bacteria in water (66). These filters have the advantage of possessing close-tolerance pores of known size and a rapid flow rate. After filtration of water or soil suspensions through the filter membrane, the organisms can be examined directly by transmitted light microscopy by first staining them directly on the membrane, then rendering it transparent by the application of immersion oil. Relatively large volumes may be quickly surveyed in this manner, possibly revealing the presence of fungus pathogens having a characteristic spore morphology (134, 135). Alternatively, the membrane may be placed in contact with a liquid or solid nutrient medium and incubated in the usual manner. However, the presence of copper, and, to a lesser extent, zinc ions reduces the number of bacterial colonies that develop (289).

DIRECT ISOLATION. Warcup (358) has isolated fungi, including Basidiomycetes, that are usually absent from dilution plates, by directly plating out mycelia visible in the soil. He prepares a soil suspension and

decants off the lighter particles several times. The remaining particles are observed under a microscope and individual hyphae or mycelial mats removed. Twenty to 50 of these are re-suspended in sterile water in a Petri dish, cooled, liquid agar poured in, and hyphae dispersed by shaking. After several days, new uncontaminated hyphae may be transferred. Saitô has used a similar technique for studying the different types of mycelia found in the soil (275).

Another, now widely used, technique, the soil-plate, minimizes the advantage that freely-sporing fungi have in the dilution plate method (37, 351). This technique involves placing soil samples directly in contact with the surface of the medium. It was first employed by Waksman in 1915 (349); later Meredith (228) modified the technique by placing the soil particles in contact with the medium for only a short time. Warcup (356) prepared soil plates by first adding liquid medium to the soil particles, then dispersing them throughout the plate before the medium had solidified. He also plated out the particles which remained intact after the soil was suspended in water. Both methods resulted in the isolation of a larger number of fungus species than with the conventional dilution plate method. Other techniques have been introduced which favor fungi having a relatively rapid linear growth rate in the soil. Chesters (52, 53) has described a glass immersion tube having several small openings through which mycelia can grow and colonize the medium which is contained in the interior. Plastic tubes, wrapped with tape to keep the contents sterile before use (239), steel tubes (336), and capillary tubes (215) have also been used. Depression slides (191, 287), placed face to face, allow rapidly growing fungi to invade a nutrient medium. Screens (335) or plastic discs (374), placed over a medium on a plate, are likewise effective in trapping soil fungi. The so-called "sugar fungi" are isolated by inserting, into soil, sterile slides one atop the other in contact at one end, but separated at the other end by a glass fiber (92). It has become apparent that the efficiency of such apparatuses depends, in part, upon their ability to alter the soil environment, for, due to fungistasis in soil, the spores of many fungi will not germinate *in situ* unless the normal soil environment is upset (92).

A direct comparison of several of these methods has shown that the immersion tube, the screened immersion plate, and direct isolation methods give essentially the same results (54). However, a greater range of species was obtained when using the screened immersion

plate method for isolation. It was felt that this was due to the presence of large apertures, which may lessen the competition for entry, the maintenance of relatively high oxygen tension, and the prevention of organisms growing under one aperture from affecting other organisms under nearby apertures.

Ectotrophic mycorrhizal fungi have been isolated directly by plating out surface-disinfested rhizomorphs taken from roots (202). When plated out, roots taken directly from the soil and dissected, so as to divide them into outer and inner portions, may yield endophytic fungi which grow out from the interior sections free of root-surface contaminants (147, 348). Some algae produce non-motile endogenous cells. As long as these remain enclosed within the cell wall of the parent plant, they are free from bacteria. New colonies can be started from these endogenous cells by first thoroughly washing the parent plant, then teasing out and transferring the cells to a suitable medium (282.)

Rather than isolating from roots immediately after collection, it is sometimes advantageous to allow reproductive or resting structures to form *in situ* and then to isolate from these. Surface-sterilized roots incubated for two to four weeks in moist, sterile sand form resting or fruiting bodies in and on the roots which can be observed and isolated by dissection. Sterile seeds or bits of plant material placed near the roots in the sand may serve as traps for certain fast-growing fungi (368). Acrasiales can be isolated by transferring sori, which have been induced to form in the soil, to plates containing a known bacterium (181). The techniques for the isolation of single cells have been reviewed by Hildebrand (158, 159) and have been quite useful for isolating organisms such as algae (31, 282), protozoa (246), or fungi (102), when single motile cells are produced which eventually grow into mature individuals.

AERATION. The ability of many fungi to grow at reduced oxygen tensions has been utilized in allowing them to grow through agar films (40, 296) or from under glass discs (220) or rings (11, 265) and so escape contaminants. An adequate oxygen supply appears necessary for the emergence of nematodes from roots (50, 364).

Oxygen may be removed by one of several techniques when anaerobic organisms are to be isolated. Evacuation, which may be followed by replacement of the atmosphere with oxygen-free gases, combustion, exclusion, exhaustion, absorption, or a combination thereof, are the most common methods employed. The procedures for obtaining condi-

tions suitable for the growth of anaerobes have recently been reviewed by McClung and Lindberg (216).

TEMPERATURE. Although organisms live within a relatively narrow temperature range, there is a spread of some 70°C. in the optimum temperature for growth of the various soil microorganisms. Togashi has listed the response of many fungi to temperature (337). Incubation at high temperatures has been used for encouraging the growth of human pathogens (37°C.) or thermophils (45–75°C.) (354). Indeed, some thermophils are unable to carry out their vital processes below 30°C. Unwanted organisms which do not produce some type of resistant structure may be eliminated by heat. Thus, protozoan cysts have been surface sterilized by heating at 37°C. for six weeks (249) or 60°C. for periods of 40 minutes to several hours (249, 339). Submersion of soil and roots in water at 75°C. for 1–1½ minutes has yielded a *Penicillium* sp. parasite of *Rhizoctonia solani* (32). Partial steam sterilization of soil at 100°C. for 2–4 minutes has permitted the isolation of Ascomycetes rarely found by other means (357). Such heat treatments appear to drastically change the overall balance of soil microorganisms (334, 352, 353). It may be that the success of this method depends primarily upon such a shift taking place, so that organisms antagonistic to the Ascomycetes are killed. On the other hand, the effect of the heat may be directly on the Ascomycetes. In some cases it has been shown that a short heat treatment is necessary to induce the germination of fungus or bacterial spores (81, 288).

On the other hand, many organisms, especially food-destroying fungi and marine bacteria, require low temperatures, many even forming colonies below 0°C. (28, 196, 379).

PRESSURE. Barophilic bacteria can grow readily under pressures of 700 atmospheres or more (380). In general, bacteria from deep-soil levels will tolerate higher pressures at higher temperatures. Many of the deep-sea bacteria are obligate barophiles. However, ZoBell has isolated bacteria in certain deep-sea sediments which will not grow at high pressures.

RELATIVE HUMIDITY. The minimum relative humidity at which a fungus is able to grow is related to the osmotic pressure of its cytoplasm (150), and, therefore, organisms tolerant of low relative humidity can usually grow on media with a high osmotic concentration. Although this fact has been utilized when isolating fungi from stored grain (59),

it does not appear to have been exploited in the isolation of soil microorganisms.

RADIATION. The effect of gamma radiation upon various micro-biological groups has been studied by Lawrence et al. (192). Their findings indicate that, in general, spore-forming aerobic bacteria are much more resistant than are either non-sporing bacteria or fungi. Yeast and protozoa also appear to be relatively resistant to gamma radiation. Protozoa have been reported to be more resistant than bacteria to X-rays (39). Exposure of nematode eggs to ultraviolet light for 30 seconds, after soaking in chlorox and washing in sterile water, has yielded sterile nematodes (38). Algae have also been subjected to uv radiation. Bacteria, harbored in the gelatinous sheath of some blue-green algae, when exposed to 275 mμ irradiation for one to 20 minutes, were killed (4). Diatoms, on the other hand, appear to be quite sensitive to uv irradiation (381). It would appear that in selected instances radiation may be helpful in eliminating surface contamination.

AGGLUTINATION. Amoss and Poston (5) were able to isolate *Brucella abortus* from a saline solution by agglutinating the bacteria with immune serum. The precipitate was centrifuged and the centrifugate placed on eosin-methylene blue agar. The results of using standard methods for bacterial isolation were always negative. This method would appear to be useful for isolating bacteria in instances where they are few and are quickly overgrown by other organisms in culture.

POLARIZATION. Amster (6) was able to separate protozoa from bacteria by repeatedly electrophorescing the organisms which migrated to the cathode. In his experiments the bacteria tended to migrate to the anode and protozoans to the cathode; however, later work has shown that the net charge of bacteria may vary with the species or strain and the pH (49, 213). A recent method, still on its development, for use with biological material, involves electrophoresis combined with other types of gradients (185).

Chemical Methods

ANTIBIOTICS. The addition of various antibiotics to the isolation medium is becoming increasingly commonplace. Several review articles have been written dealing with antibiotics in general (23) and those produced specifically by fungi (36) or Actinomycetes (26). New materials in relation to their effect on microorganisms are often discussed

in the journal *Antibiotics and Chemotherapy*. The tolerance of organisms to new antibiotics can be tested easily by the use of single-gradient plates (42, 321, 322). This method involves first allowing a layer of agar to harden in a Petri dish which has been slanted so that the medium just reaches to the highest edge. After placing the dish in a horizontal position, a second layer, containing the antibiotic, is added. The drug diffuses downward resulting in its being diluted in proportion to the thickness ratio of the two layers. A uniform concentration gradient is set up across the plate. Double-gradient plates may also be used which allow one to vary the concentration of two materials, each material being incorporated into one of the two layers (363).

A combination of streptomycin and penicillin appears to be the most extensively used and has aided in the isolation of bacteria-free diatoms (306), Myxomycetes (303, 304, 362), nematodes (95, 162), algae (204), amoebae (293), and marine Phycomycetes (347, 362). Streptomycin has also been used alone (146, 208, 209, 238, 280), as has penicillin (116). Aureomycin has been used for isolating yeasts (155) and fungi (75, 147). Novobiocin eliminates soil bacteria and reduces the radial growth of fungi; however, certain species of *Pythium* and *Phytophthora* are completely inhibited at the concentration used, 100 ppm (44, 160). Sterile amoebae have been obtained by the use of terramycin or erythromycin (146). Muller has reported on the effect of nine antibiotics on a series of plant pathogenic fungi and bacteria (241). Mixtures, such as polymyxin B, bacitricin, and circulin for isolating *Brucella* spp. from the soil (189), pimaricin, penicillin, and polymyxin for *Phytophthora* spp. (101), or subtilin, penicillin, actidione, and polymyxin for isolating *Streptomyces* spp. (98), have been used. It has been suggested that polymyxin B (121), as well as a number of other antibiotics (165), could be used to eliminate bacteria, fungi, and other algae as well, from algal cultures.

For the suppression of saprophytic fungi cycloheximide, or actidione, is usually incorporated into the culture medium. It not only has low activity against plant pathogenic fungi (163, 280), but is innocuous to fungus pathogens of animals (119), Actinomycetes (77), and bacteria (168, 258) as well.

SURFACE STERILANTS. Because of the inherent resistance of resting structures, they can withstand a relatively harsh surface disinfestation; solutions containing sodium hypochlorite or mercuric chloride are the

most widely used. A general treatise on the properties of the various groups of common disinfectants has been written by Rahn (264). Amoeba cysts have been successfully surface-sterilized with saturated sodium carbonate or concentrated sodium chloride solutions, toluene, chlorine water, calcium hydroxide, silver nitrate, or calcium sulfide (118, 286), and nematodes and their eggs with hydrogen peroxide (45, 342), merthiolate (95), sodium hypochlorite (38, 127), thiocarbamate fungicides (162), ethoxy ethyl mercury chloride (211), or mercuric chloride (45). Nematode eggs are sometimes collected from within the bodies of gravid females, which have been killed and surface sterilized with strong chemical agents (305). Heavily contaminated materials containing dermatophytes have been treated with commercial fungicides (123). Potassium dichromate has been used in the sterilization of flagellates (128, 129). Sclerotia may be surface-sterilized in much the same manner as plant parts; the larger ones can even be dipped briefly in alcohol and quickly passed through a flame. Rhizomorphs have been surface-sterilized by washing for ten seconds in 0.1 per cent hydrochloric acid (202). Soaking roots or potato tubers in a combination of calcium hypochlorite and sodium hydroxide has been used when attempting to isolate Actinomycetes (327). Davies found a one per cent silver chloride solution was less toxic to *Ophiobolus graminis* and more toxic to *Helminthosporium sativum* than was mercuric chloride (85). It follows that inducing the formation of resistant structures by physical or chemical means would permit employment of these more rigorous sterilization procedures (127, 128).

SURFACE-ACTIVE AGENTS. When making suspensions, clumps of nonwetting fungus spores can be wetted and separated from each other by the addition of sodium lauryl sulfonate (332). Tween 80 (polyoxyethylene sorbitan monooleate) has been used for the same purpose with bacteria (309). It is also non-toxic to some fungi up to a concentration of one per cent (325). Synthetic detergents are also bactericidal, cationic detergents being more active against gram positive than gram negative bacteria, while anionic detergents are only moderately active against gram positives and are ineffectual with gram negative bacteria (19, 131). The pH is quite important in determining efficiency (124), cationics being more active in the alkaline range and the anionics in the acid range (18). Dehydrated oxgall suppresses the growth of saprophytic fungi (208, 209), Actinomycetes (229), and bacteria (20,

230, 256). It compares favorably with either penicillin G or streptomycin and is better than crystal violet in suppressing growth of soil bacteria (20).

DYES. Many dyes are bacteriostatic (41, 184). Gentian violet and crystal violet are selectively inhibitory to gram positive bacteria (62), while acid fuchsin or neutral acriflavine are active against gram negatives (63, 64). Crystal violet (189, 209, 256) or rose bengal (224, 298) has been used for the isolation of fungi or bacteria, acriflavine for diatom (381) or dermatophyte (310) isolation, and malachite green for obtaining bacteria-free nematodes (149). The inhibition of growth of *Fusarium* spp., *Pythium ultimum,* and *Rhizoctonia solani* by various dyes has been studied by Munro and Newton (242).

SYNTHETIC MEDIA. An extremely wide range of synthetic culture media has been formulated since the pioneering work of Raulin in 1869. A compilation of these up to 1926 lists some 7,000 preparations suitable for microorganisms (201). The historical development of culture media suitable for marine algae has been traced from the 1890's to the present (262). Media for the axenic culture of invertebrate metazoa have also been reviewed (94). Detailed chemical analyses of some of the large selection of commercially prepared dehydrated media (21, 91) available have been compiled (43, 320).

It should be kept in mind that some taxonomic groups possess specialized enzyme systems capable of utilizing for a carbon source compounds such as: cellulose (294), sodium benzoate (166), creosote (222), paraffin (136), and even such a compound as Tween 80 (234). These groups can be isolated by using a medium "baited" with the appropriate carbon source. Such a technique has been called "enrichment culture," a much-used technique of Beijerinck's in the late 1800's. This method, an application of the principle of competitive exclusion, is not without limitation, however, since the preferential multiplication of one organism in a natural system will tend in time to make the environment less favorable for that organism, resulting increasingly in the multiplication of other species. This ecological succession within the system makes vitally important the time at which isolation from the bait is attempted.

Organisms may also be able to utilize only certain types of nitrogen or sulfur sources (117), and in some cases one or more vitamins or "growth factors" must also be added to the medium to permit growth to take place. In addition, autotrophs may require some oxidizable com-

pound such as: hydrogen gas, carbon monoxide, methane, or reduced forms or iron or sulfur for growth.

Although agar (161) is almost universally used as the solidifying agent for the isolating medium, other materials such as gelatin, silica gel (180, 260), porous clay cups (210), plaster of Paris, sand, gypsum, glass wool (30, 200), cellophane (144), or molecular membrane filters (84, 373) have been employed in special cases.

HYDROGEN-ION CONCENTRATION. In general, fungi are able to grow more readily at a lower pH than either bacteria or Actinomycetes. Advantage was taken of this fact when Waksman in 1922 proposed acidifying the medium with lactic, phosphoric, or sulfuric acid in the dilution-plate technique for isolating soil fungi (350). Other materials used to reduce the pH have been boric (343), citric (247), humic (88), or picric acid (14) or potassium di-hydrogen phosphate (295). A simplified method, homologous to the single-gradient plate technique, allows a pH gradient to be set up in a single Petri dish (274). It should be borne in mind that some organisms will grow only over a relatively narrow pH range and that the pH of the medium may change rapidly, if not properly buffered, due to their metabolism.

OXIDATION REDUCTION POTENTIAL. An Eh favorable for the growth of aerobic organisms is usually part of the isolation environment. Cultivation of anaerobes thus requires providing a favorable O/R potential (268).

MISCELLANEOUS CHEMICALS. The differential tolerance of microorganisms to toxic chemicals, as well as to heat, could be used as an aid in isolation, if the organisms desired are comparatively resistant to the chemical used (110, 139, 225, 344). Such chemicals, when incorporated into the isolation medium in suitable concentrations, will allow selective growth of the organisms in question. Small dosages of chloropicrin applied to the soil reduce fungi, but virtually eliminate bacteria and protozoa (297). Fungi show a wide variation in their tolerance to the vapors of diphenyl (151) or propylene oxide (252). Mold and bacterial growth has been inhibited by using sodium or calcium propionate when isolating Actinomycetes (80). Later work with yeasts has shown that the use of propionate or diphenyl is limited (109). Saprophytic fungi, especially *Trichoderma viride*, but not wood-rotting Basidiomycetes, are suppressed on a medium containing *o*-phenyl phenol (164). Addition of sodium dichromate to the medium allows *Corynebacterium sepedonicum* to be isolated from potatoes in the presence of

soft-rot bacteria (299). Phenol as a treatment of potatoes prior to isolating Actinomycetes has been shown to be superior to sodium propionate (193). Soil treated with hydrochloric acid kills active protozoa but cysts remain viable (82). Sorbic acid selectively inhibits catalase-positive microbes. It has been suggested that tetrazolium compounds could be used in the isolation of human pathogens (279).

Biological Methods

BAITS. Leeuwenhoek in 1676, unwittingly, was probably among the first to use baits as a means of attracting and concentrating organisms from the surrounding environment (93). This technique is in reality not unlike "enrichment culture." Here the living host or organic material used as the bait constitutes the enrichment medium. In many cases pure cultures can readily be obtained by culturing directly from the bait after it has been in contact with infested soil, water, or plant material for a period of time (103).

Examples of this are: carrot disks (375) or tobacco seedlings (126) for *Thielaviopsis basicola;* rose stems for *Chalaropsis thielavioides* (17); carrot disks and other fleshy roots for crown gall (12) and hairy root (13); potato tubers for soft-rot bacteria (176), *Fusarium caeruleum* (217), blackleg bacteria (194), and *Phytophthora infestans;* turnips for *Phoma lingam* (125); pears for *Erwinia amylovora;* skin, wool, or hair for kerotinophilic chytrids (174) and dermatophytes (1, 345, 346); chitin for chitinophilic chytrids (173); panels of different kinds of wood, rope, or cordage for marine fungi (22, 170, 243) or wood rotters; straws for root-infecting fungi (359); autoclaved wool or cotton for fabric-decomposing organisms (366); rice grains for *Chromobacterium* spp. (78); hemp seed for Saprolegniaceae (245, 333); avocado (377), apple (47, 340), or pineapple shoots or roots (7) for *Phytophthora cinnamomi;* lemon or orange fruit for *Phytophthora citrophthora, P. parasitica,* and *P. syringae* from soil and diseased material (182, 183); viable sclerotia for soil organisms which are able to colonize sclerotia (114); and living hosts of particular plant pathogens (107, 122, 257, 277, 300, 301, 367). In this connection it has been suggested that fruit and seed are infected mostly by the more invasive clones of an organism (16). Other baits have been used for aquatic Phycomycetes (79, 102) and predaceous fungi (96).

In the field of medical mycology animal injection has proven to be of value in isolation. A soil suspension, suspected of containing the

pathogen, is introduced into a test animal, which, after a certain time, is sacrificed (361). The pathogen, if present, can usually be isolated in pure culture from the appropriate tissue (2, 46, 72, 105, 106, 240, 269).

NATURAL MEDIA. Some fungi will produce reproductive structures and grow more profusely when grown on natural plant or animal tissues or extracts from them (70, 173, 231, 302). Such materials, when sterilized with propylene oxide at room temperature, rather than autoclaved, remain in a more natural state and support better growth (141). A number of species of free-living protozoa can be maintained on media made from organic materials (246).

OBSERVATIONAL TECHNIQUES

STAINING. Valuable adjuncts to isolation are the methods for examining the soil microorganisms *in situ* by direct microscopy. Conn (73, 74) seems to have been the first to advocate this method. His procedure consisted of making a soil suspension in dilute gelatin, drying this on a slide, and staining it before examination. Rose bengal or erythrosin in five per cent aqueous phenol was used since they do not stain the soil colloids. Later workers have effectively used other stains specific for organisms, such as fast acid blue R (271), methyl green (329), cotton blue (236), and acridine orange which is used in conjunction with a fluorescent microscope (318, 319). Tchan has utilized fluorescent microscopy for studying soil algae (329, 330).

A number of workers have successfully used the fluorescent antibody technique to stain specific microorganisms, and it appears that this technique would also be a useful tool to those engaged in observing soil microorganisms. The technique makes use of the fact that antibodies produced in animals in response to the microorganism or extract therefrom, when conjugated with fluorescein isothiocyanate, react rather specifically with the microorganism. Examination with a fluorescent microscope shows up the microorganisms stained with the fluorescent antibodies (223).

Winogradsky (371, 372) amplified and improved Conn's technique by first allowing the large soil particles in the suspension to settle. These particles were washed and the washings and remains of the original suspension combined, and centrifuged. Aliquots from both the supernatant and centrifugate were then dried on slides and stained. One disadvan-

tage to this technique is that filamentous forms are so broken up as to make many of them unrecognizable.

Soil may also be ground with water and mixed with liquid agar on a haemocytometer slide (172); this gives a uniformly thin film which may be floated off and stained after the agar solidifies. In this method more filamentous organisms remain in their normal state.

DIRECT OBSERVATION. Direct examination of fragments of undisturbed soil (186, 187, 188), using reflected light and a specially designed soil microscope (187), has given the most accurate picture of the soil's normal appearance. This method has also been applied in a quantitative sense to the study of nematode populations in soil (311).

CONTACT SLIDE. Perhaps the best known of the observational methods is the Rossi-Cholodny glass slide technique. As originally developed by Rossi (272), it consisted of pressing slides against an undisturbed vertical soil face; after removal and staining, a picture of the organisms as they actually occurred in the soil was obtained. Cholodny (56) modified this by allowing the slides to remain in the soil for five days to three weeks before removal. Further modifications have been proposed (54, 76, 132, 273). The technique is applicable to both qualitative and quantitative investigations (30).

An analogous procedure has been employed by Henrici (152) to study bacteria in fresh water streams. He has also observed algae and protozoa by this method. The technique had been used earlier by various workers in studying specific biological groups, such as the diatoms (333), iron bacteria (55, 245), or sessile aquatic microorganisms (153, 214).

SOIL CHAMBER. Cholodny has also studied soil organisms in their natural habitat by constructing small transparent soil chambers (57). The chambers, in reality small moist chambers, are prepared using a specially made soil press which forms a slice one mm in depth with a four mm hole through it. The soil slice rests on a glass slide while the top of the slice is covered over with a cover glass to reduce drying. Continuous observation of bacteria, fungi, Actinomycetes, and protozoa which develop on the cover glass is possible.

Another type of soil chamber (58) which he has utilized is made by sieving soil onto a cover slip and inverting it over a depression slide containing a few drops of water. Colonies can be observed around and on the individual soil particles after several days.

Pieces of glass, slides, or even cover slips have been incorporated

into sides of boxes containing soil in which plants can be grown. Roots growing against the glass surface may be examined under high magnification using reflected light (205, 207). Large boxes have been constructed which allow both observation and periodic removal of the slide, so that roots may be removed and cultured (71, 87). Dean (87) has said of this method, "parasites which would have wholly escaped us by the usual method of pulling up sickly plants in the field have been discovered at work and their progress observed in detail."

Den Ouden has described an observational method which consists of allowing the plant's root system to develop in a thin layer of foamed agar enclosed in a flat polyethylene envelope (89). Microorganisms can be introduced under the polyethylene at suitable positions and their effect on the whole root system observed.

SOIL SECTIONING. Thin sections of soils may be prepared and microscopically examined by transmitted light. Standard rock or plant microtechniques for sectioning are utilized and attempts made to keep the soil and its associated organisms as nearly as possible in their natural state. The soil sample may be stained before impregnation with the solidifying medium.

Haarlφv and Weis-Fogh (138) have developed a method using two per cent agar as the hardening agent for litter. This method is, however, unsatisfactory for mineral soils. Later workers have used resins (3, 154) as a hardening agent. Minderman (235) has impregnated soil samples with gelatin after freezing, then dissolved the silica with 50 per cent hydrofluoric acid. Sections 7.5–10 μ in thickness can then be cut on a microtome. Pedologists have also used resins (86), plastic (33), lacquer (145), cellulose (283), and paraffin (259) as hardening agents when studying soil structure. Plastics and resins, however, have a drawback in that they dehydrate animal tissues (188).

While all these observational methods have their value, none quite achieves the ideal goal—the ability to observe happenings as they would occur under natural conditions over an extended time period.

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FACTORS AFFECTING THE FREQUENCIES OF WILD PLANT HYBRIDS

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INTRODUCTION

The frequencies with which wild plant hybrids occur differ markedly from place to place and from genus to genus. For example, amongst the orchids wild hybrids between certain *Ophrys* species are rare (Stebbins and Ferlan, 1956), whilst wild hybrids between certain *Odontoglossum* species are abundant (Rolfe, 1908). It is obvious that the frequencies of wild hybrids are controlled by many factors, but little is known about them. It was long ago recognised that species which do not produce hybrids in nature, might do so under garden conditions. In the middle of the eighteenth century Gmelin (see Zirkle, 1935) noted that two species of *Aquilegia* hybridized readily in his garden but that wild hybrids between them were not known. He attributed the absence of wild hybrids to the differing ecological and hence differing local geographical distributions of the parent species. A wide range of examples similar to *Aquilegia* have been analyzed by Anderson (1949) who has given reasons based upon genetic theory to ex-

plain why intermediate and disturbed habitants promote the survival of wild hybrids.

In this paper the relationships between hybrid frequency and a few biological properties of the parent species and their artificial F_1 hybrid will be considered to determine whether any significant correlations between these properties and hybrid frequency can be demonstrated. The three features of the parent species chosen for study were their breeding behaviour, levels of ploidy and life forms. Besides these, the relationship between the fertility of artificial F_1 hybrids and the frequencies of wild hybrids has been investigated.

The data for analysis have been taken from the literature and are summarized in Table VII. There has been no selection of data, and the examples quoted include all those met with in the literature consulted. Estimates of wild hybrid frequencies have been divided into two categories, rare and common. Hybrids have been regarded as common if they are anywhere abundant, and hybrids rare in one locality but not another have therefore been listed as common. There are few numerical estimates of hybrid frequency available, and the remainder are quite subjective. It was anticipated that some degree of objectivity would be obtained if the estimates of hybrid frequencies were reduced to one of two categories.

Each species occupies a separate line of Table VII. This enables the data for each to be listed separately. For most species the data have been collected from several sources, and the appropriate authorities are listed collectively for each species cross. Some of the Table entries, especially those concerning hybrid frequency and life forms, are the writer's interpretation of the original data where these were not suitable for direct entry in the Table.

The relationship between hybrid frequency and each of the four variables studied will now be considered.

HYBRID FREQUENCIES AND THE BREEDING MECHANISMS OF THE PARENT SPECIES

Self-sterile species are obligatory outbreeders, and so it would be expected that such species would be involved more frequently in the production of wild hybrids than self-fertile species. It seemed likely, therefore, that there would be some correlation between the self compatibility or otherwise of a species and the frequency of wild hybrids derived from

it. That this is not so, however, may be seen from Table I. In this Table there is close agreement between the observed and expected

TABLE I

EXAMPLES OF RARE AND COMMON WILD HYBRIDS, CLASSIFIED ACCORDING TO THE SELF FERTILITY OR OTHERWISE OF THEIR PARENT SPECIES. FIGURES IN PARENTHESES ARE THE EXPECTED VALUE.

Parent Species	Frequencies of Wild Hybrids	
	Rare	Common
Both self fertile	11 (10.7)	19 (19.3)
One or both self sterile	9 (9.3)	17 (16.7)

numbers for each entry of the Table. The expected values in this and subsequent Tables have been calculated on the basis that there is no correlation between the pairs of characters under consideration. The method of calculating these expected values may be found in most textbooks of statistics.

Results similar to the above were obtained by Allan (1931) from a study of wild hybrids in the New Zealand flora. There he classified genera of two or more species into three classes—hermaphrodite, monoecious and dioecious. The genera were further classified into groups on the basis of whether or not they possessed hybrids. A statistical analysis of his data (1931, p. 446) shows no evidence of correlation between arrangement of the sexes in the genera and the occurrence of wild hybrids.

WILD HYBRID FREQUENCIES AND THE PLOIDY LEVEL OF THE PARENT SPECIES

Hybrids between homoploid species are usually easier to make than those between species with different chromosome numbers. It therefore seemed likely that wild hybrids between homoploid species would occur more abundantly than hybrids between heteroploid species. As may be seen from Table II, there is a tendency for this to be so, and both common wild hybrids derived from homoploid species and rare wild hybrids derived from heteroploid species occur in excess of their expected numbers. However, the differences between the observed and the expected values are not significant (5% level), and so the data cannot be regarded as conclusively demonstrating a definite associa-

tion between the ploidy of the parent species and the frequencies of their wild hybrids.

TABLE II

EXAMPLES OF RARE AND COMMON WILD HYBRIDS, CLASSIFIED ACCORDING TO THE PLOIDY OF THE PARENT SPECIES. FIGURES IN PARENTHESES ARE EXPECTED VALUES.

Parent Species	Frequencies of Wild Hybrids	
	Rare	Common
Homoploid	11 (14.5)	32 (28.5)
Heteroploid	10 (6.5)	9 (12.5)

WILD HYBRID FREQUENCIES AND THE LIFE FORMS OF THE PARENT SPECIES

Under experimental conditions artificial F_1 hybrids are often more vigorous than their parents; so, if these hybrids maintain their vigour under field conditions and are also capable of vegetative reproduction, it would be anticipated that they would accumulate in populations at the expense of their parent species. Hybrids incapable of vegetative reproduction would have less chance of accumulating.

Assuming this argument to be valid, there should be a marked correlation between hybrid frequencies and their capacity for vegetative reproduction. Reference to Table III will show that no such correlation exists. There is close agreement between the observed numbers of examples in each group and those expected, assuming there to be no correlation between the frequency of wild hybrids and their capacity for vegetative reproduction. Data on capacity for vegetative reproduction are based upon personal experience of the plants or on reference to a wide range of regional floras.

TABLE III

EXAMPLES OF RARE AND COMMON HYBRIDS, CLASSIFIED ACCORDING TO THE ABILITY OF THEIR PARENT SPECIES TO REPRODUCE VEGETATIVELY. FIGURES IN PARENTHESES ARE EXPECTED VALUES.

Reproduction of Parent Species	Frequency of Wild Hybrids	
	Rare	Common
Non vegetative	10 (9.2)	18 (18.8)
Vegetative	13 (13.8)	29 (28.2)

When discussing hybrid frequencies in natural populations, Stebbins (1950) suggested that perennial hybrids might be expected to occur more frequently than annual hybrids because with a perennial a favourable hybrid segregate will be able to survive longer than will an annual. It therefore seemed appropriate to compare the frequencies of different Raunkaierian life forms (Raunkaier, 1934) in examples of hybrids classified as rare or common in the Appendix. This comparison may be made from Table IV, from which it is clear that there is no correlation between life form and hybrid frequency.

TABLE IV

EXAMPLES OF RARE AND COMMON WILD HYBRIDS, CLASSIFIED ACCORDING TO THE LIFE FORMS OF THE PARENT SPECIES. FIGURES IN PARENTHESES ARE THE EXPECTED VALUES.

Hybrid frequency	Life Form					
	Th	H, Hel, Hyd.	Ch	N	M, MM	E
Rare	7(5.3)	10(11.5)	2(1.6)	1(1.6)	3(2.6)	0(0.3)
Common	9(10.7)	25(23.5)	3(3.4)	4(3.4)	5(5.4)	1(0.7)

WILD HYBRID FREQUENCIES AND THE FERTILITIES OF THEIR ARTIFICIAL F_1 GENERATION

Since fertile F_1 hybrids are capable of producing further hybrid offspring and sterile F_1 hybrids are incapable of producing secondary hybrids, it was thought that the frequencies of wild hybrids might be related to the fertilities of their artificial F_1 generation.

To test this view the average fertilities were calculated for the F_1 generations of the hybrids classified as rare or common in Table V. When calculating the average fertilities, only those F_1 hybrids for which a numerical estimate of fertility was available were considered, as the terms fertile and sterile are often loosely employed without being qualified by information on the degree of fertility or sterility involved.

TABLE V

AVERAGE FERTILITIES OF THE ARTIFICIAL F_1 GENERATION FOR WILD HYBRIDS, CLASSIFIED AS RARE OR COMMON.

Hybrid Frequency	Percentage Fertility					
	Seed			Pollen		
	n	m	range	n	m	range
Rare	6	10.9	1–25	10	48.2	4–94
Common	12	31.4	0–94	22	44.3	0–96

From Table V there appears to be a difference in the average seed fertilities of the F_1 generations of hybrids which are rare or common in the field, but this difference is not statistically significant (5% level). The average pollen fertility of hybrids classified as rare or common is similar, but these data are not as reliable as those for seeds, since the pollen viability is usually estimated by staining and not by germination of the grains.

DISCUSSION

It had been anticipated that the frequencies of wild plant hybrids would be correlated with each of three selected properties of their parent species and the fertility of their artificial F_1 generation. Yet from the above analyses it is clear that should such correlations exist, the data are insufficient to demonstrate them with the techniques employed.

There appeared to be a weak correlation between the fertility of the artificial F_1 generation and wild hybrid frequencies, but more data are requred to determine its valadity. The slight correlation observed between ploidy of the parent species and the frequencies of the wild hybrids is probably a reflection of the correlation noted between the fertility of the F_1 generation and wild hybrid frequency, since hybrids between heteroploid species are in general more likely to be sterile than hybrids between homoploid species.

Some of the reasons for the apparent lack of correlation between wild hybrid frequencies and the biological properties of the parent species and F_1 generation will now be considered.

The need for intermediate and disturbed habitats for the successful growth of hybrids has been mentioned above. Suitable habitats of these types are often transient, and in any one district the frequencies of wild hybrids may fluctuate according to the size and number of available habitats. There is a considerable body of data showing that disturbance of the habitat leads to an increase in the number of natural hybrids in a district, but there are few data on hybrid frequencies within communities returning to equilibrium after disturbance.

Nevertheless the available data show that hybrid frequencies in such developing communities do not necessarily remain constant. Sometimes, as in *Viola* (Clausen, 1926), the hybrids tend to die out as the community matures. At other times, as the community develops, there may be an increase in the frequency of hybrids relative

to that of the parent species. This has been noted for some *Dactylorchis* hybrids which, along with other plants, were colonizing a disused clay pit (Richardson, 1957). Here was a five-fold increase in the proportion of wild hybrids in the *Dactylorchis* population during a five-year period. The manner in which the proportions changed during this time are shown in Table VI.

TABLE VI

THE PERCENTAGE OCCURRENCE OF TWO *Dactylorchis* SPECIES AND THEIR WILD HYBRID OVER A PERIOD OF FIVE YEARS (DATA FROM RICHARDSON, 1957)

Taxon	Percentage of Individuals			
	1949	1951	1953	1954
D. fuchsi	41	30	15	14
D. purpurella	44	22	5	4
hybrid	15	48	80	82

Apart from long-term changes in hybrid frequencies there may be fluctuations from season to season, as has been described for the genus *Delphinium* (Epling and Lewis, 1952). Here the hybrids are perennial and do not necessarily flower annually. As they are mostly recognised in bloom, the majority will be overlooked in unfavourable flowering seasons.

Besides varying from season to season in one locality, hybrid frequencies may differ considerably from one locality to another within the ranges of the parent species. For example, whereas hybrids between *Primula vulgaris* and *P. veris* are uncommon in England (Clifford, 1958), they are reported to be abundant in northern Italy (Pugsley, 1927). Similarly, hybrids between two species of *Acer* are uncommon in the northern United States (Anderson and Hubricht, 1938) but are relatively common in the Laurentian Peninsula of Canada (Dansereau et Lafond, 1941).

From the foregoing discussion it is clear that estimates of hybrid frequency have little meaning unless accompanied by a good deal of qualifying information. The failure of the techniques employed above to detect correlations between hybrid frequencies and the other variables studied has probably arisen from classifying hybrids as rare or common without considering data other than that being classified. It is hoped

that it will be possible at some future date to refine the analysis by considering both the status and frequency of hybrids in populations but such an analysis awaits the production of further data.

SUMMARY

The relationships between the frequencies of wild hybrids and certain properties of the parent species and the artificial F_1 generation have been investigated from a survey of the literature. There appears to be no correlation between hybrid frequency and the life form of parent species, neither is there any correlation detected between hybrid frequency and the self compatability or otherwise of the parent species. There appears to be a weak correlation between hybrid frequency and the fertility of the artificial F_1 generation; also between hybrid frequency and the level of ploidy of the parent species; but neither of these correlations is statistically significant.

TABLE VII

PERCENTAGE OF FERTILITY, FREQUENCY AND OTHER DATA REGARDING WILD SPECIES HYBRIDS

Species Cross	n	Fertility percentage of F_1 generation		Hybrid frequency in population	Compatibility of parent species	Life form of parent species	Authority
		Pollen	Seed				
Agropyron scabrum ×	21	33	—	rare	self fertile	H	Connor, 1954, 1956
A. kirkii	21				self fertile	H	
Agropyron enysii ×	21	0	0	common	self fertile	H	Connor, 1954, 1956
A. scabrum	21				self fertile	H	
Agrostis tenuis ×	14	14	2	common	self fertile	H	Bradshaw, 1958
A. stolonifera	14				self fertile	H	
Aloe striata ×	7	fertile		common	———	Ch	Warren, 1931
A. saponaria	7				———	Ch	
Amaranthus dubius ×	32	2	—	common	self fertile	Th	Clifford (unpub.)
A. spinosus	16				self fertile	Th	
Amsinckia glariosa ×	12	fertile		common	self fertile	Th	Ray and Chisaki,
A. tessellata	12				self fertile	Th	1957
Amsinckia intermedia ×	19, 15	—	6	common	self fertile	Th	Ray and Chisaki,
A. lycopsoides	15				self fertile	Th	1957
Aster novae-angliae ×	5	partly fertile		common	self fertile	H	Wetmore and
A. multiflorus	5				self fertile	H	Delisle, 1939
Aster shortii ×	36	—	25	rare	self sterile	H	Avers, 1953
A. cordifolius	18				self sterile	H	
Betula pubescens ×	14	sterile		common	self fertile	MM	Johnsson, 1945
B. verrucosa	28				self fertile	MM	
Bromus hordaceus ×	—	fertile		rare	———	H or Th	Holmberg, 1924
B. mollis	14				———		Nilsson, 1931
							Clapham et al. 1957
Bromus inermis ×	28, 14	partly fertile		common	self fertile	H	Elliott, 1949
B. pumpellianus	14				self fertile	H	

TABLE VII (Cont.)

PERCENTAGE OF FERTILITY, FREQUENCY AND OTHER DATA REGARDING WILD SPECIES HYBRIDS

Species Cross	n	Fertility percentage of F_1 generation		Hybrid frequency in population	Compatibility of parent species	Life form of parent species	Authority
		Pollen	Seed				
Centaurea nigra ×	22	fertile		common	self sterile	H	Marsden-Jones
C. jacea	22				self sterile	H	and Turrill, 1954
Centaurea nigra ×	22	fertile		common	self sterile	H	Marsden-Jones
C. nemoralis	22				self sterile	H	and Turrill, 1954
Cistus monspeliensis ×	9	10	—	common	———	N	Dansereau, 1941
C. salviifolius	9				———	N	Gard, 1912
							Darlington and Wylie, 1956
Cistus ladaniferus ×	9	20	—	common	———	N	Dansereau, 1941
C. laurifolius	9				———	N	Gard, 1912
							Darlington and Wylie, 1956
Coprosma propinqua ×	—	—	90	common	self sterile	M	Allan, 1926, 1929
C. robusta	11, 22				self sterile	M	Darlington and Wylie, 1956
Elymus wiegandii ×	14	highly sterile		rare	self fertile	H	Church, 1954
E. viparius	14				self fertile	H	
Epilobium hirsutum ×	18	partly fertile		common	———	H	Compton, 1913
E. montanum	18				self fertile	H	Akerman, 1921
							Clapham et al, 195?
Festuca pratensis ×	7	13	7	common	self fertile	H	Peto, 1933
Lolium perenne	7				self fertile	H	Jenkin, 1933
Festuca gigantea ×	21	highly sterile		rare	self fertile	H	Jenkin, 1933
F. arundinacea	21				self fertile	H	Nilsson, 1935
							Clapham et al., 1957

TABLE VII (Cont.)

PERCENTAGE OF FERTILITY, FREQUENCY AND OTHER DATA REGARDING WILD SPECIES HYBRIDS

Species Cross	n	Fertility percentage of F_1 generation		Hybrid frequency in population	Compatibility of parent species	Life form of parent species	Authority
		Pollen	Seed				
Galeopsis tetrahit ×	16	59	—	common	self fertile	Th.	Muntzing, 1930
G. bifida	16				self fertile	Th.	
Galeopsis pubescens ×	8	9	—	rare	self fertile	Th.	Muntzing, 1930
G. speciosa	8				self fertile	Th.	
Galium rubrum ×	44	90	—	common	self fertile	H	Ehrendorfer, 1955
G. pumilum	44				self fertile	H	
Geranium bohemicum ×	14	sterile		rare	self fertile	Th.	Dahlgren, 1952
G. lanuginosum	24				self fertile	Th.	
Geum urbanum +	21	78	72	common	self fertile	H	Marsden-Jones, 1930
G. rivale	21				self fertile	H	Clapham et al., 1957
							Gajewski, 1957
Glyceria fluitans ×	20	0	0	common	self fertile	H	Borrill, 1958
G. plicata	20				self fertile	H	
Gossypium arboreum ×	13	fertile		common	self fertile	Th.	Hutchinson et al., 1947
G. herbaceum	13				self fertile	Th.	Hayes et al., 1955
Helianthus divaricatus ×	17	highly fertile		common	self sterile	H	Smith and Guard, 1958
H. microcephalus	17				self sterile	H	
Helianthus annuus ×	17	19	10	common	self sterile	Th	Heiser, 1949
H. bolanderi	17				self sterile	Th	
Helianthus annuus ×	17	14	—	common	self sterile	Th	Heiser, 1947
H. debilis	17				self sterile	Th	

TABLE VII (Cont.)

PERCENTAGE OF FERTILITY, FREQUENCY AND OTHER DATA REGARDING WILD SPECIES HYBRIDS

Species Cross	n	Fertility percentage of F_1 generation		Hybrid frequency in population	Compatibility of parent species	Life form of parent species	Authority
		Pollen	Seed				
Helianthus mollis ×	17	94	—	rare	self sterile	H	Jackson and Guard,
H. grossoserratus	17				self sterile	H	1955, 1956
Helianthus giganteus ×	17	60	—	rare	self sterile	H	Jackson and Guard,
H. mollis	17				self sterile	H	1955, 1956
Helianthus giganteus ×	17	96	—	common	self sterile	H	Long, 1955
H. grossoserratus	17				self sterile	H	
Helianthus annuus × *H. argyrophyllus*	17	33	25	rare	self sterile	Th.	Heiser, 1951
Helocarpha virgata ×	4	—	1	rare	self sterile	Th.	Clausen, 1951
H. heermannii	6				self sterile	Th.	and pers. comm.
Lactuca sativa ×	9	fertile		common	self fertile	H	Thompson et al.,
L. seriola	9				self fertile	H	1941
							Anderson, 1949
Lactuca graminifolia ×	17	fertile		rare	———	Th.	Whitaker, 1944
L. canadensis	17				———	H.	
Layia platyglossa ×	7	—	1	rare	self sterile	Th.	Clausen et al., 1945
L. pentatchaeta	8				self sterile	Th.	
Lesquerella lescurii ×	8	82	94	common	self sterile	Th.	Rollins, 1957
L. densipila	8				self sterile	Th.	
Melandrium album ×	12	75	—	common	self sterile	Ch.	Baker, 1947
M. dioicum	12				self sterile	Ch.	
Nasturtium officinale ×	16	20	7	common	self fertile	H	Howard and
N. uniseriatum	32				self fertile	H	Manton, 1946
Nuphar pumila ×	17	14	—	common	———	Hyd.	Heslop-Harrison,
N. lutea	17				———	Hyd.	1953

TABLE VII (Cont.)

PERCENTAGE OF FERTILITY, FREQUENCY AND OTHER DATA REGARDING WILD SPECIES HYBRIDS

Species Cross	n	Fertility percentage of F_1 generation		Hybrid frequency in population	Compatibility of parent species	Life form of parent species	Authority
		Pollen	Seed				
Odontoglossum crispum ×	—	high		common	———	E	Rolfe, 1908
O. leptopurpureum	—				———	E	
Parthenium argentatum ×	36, 72	80	—	rare	self fertile	N	Rollins, 1944, 1945
P. mariola	81–104				self fertile	N	Powers and Rollins, 1943
Phormium tenax ×	—	fertile		common	———	Ch.	Allan and Zotov,
P. colensoi	—				———	Ch.	1937 & pers. Comm.
Pinus contorta ×	—	50	—	common	self fertile	MM	Righter and
P. banksiana	—				self fertile	MM	Stockwell, 1949; Mirov, 1956
Pinus coulteri ×	—	70	—	rare	self fertile	MM	Zobel, 1951 and
P. jeffreyi	—				self fertile	MM	pers. comm.
Pinus attenuata ×	—	91	—	rare	self fertile	MM	Stockwell and
P. radiata	—				self fertile	MM	Righter, 1946
Populus alba ×	18	fertile		common	self sterile	MM	Peto, 1938
P. grandidentata	18				self sterile	MM	Johnson and Heimburger, 1946
Populus alba ×	18	fertile		common	self sterile	MM	Peto, 1938
P. tremuloides	18				self sterile	MM	Johnson and Heimburger, 1946
Primula vulgaris ×	11	32	—	common	self sterile	H	Pugsley, 1927
P. veris	11				self sterile	H	Valentine, 1955 Clifford, 1958

TABLE VII (Cont.)

PERCENTAGE OF FERTILITY, FREQUENCY AND OTHER DATA REGARDING WILD SPECIES HYBRIDS

Species Cross	n	Fertility percentage of F_1 generation		Hybrid frequency in population	Compatibility of parent species	Life form of parent species	Authority
		Pollen	Seed				
Primula vulgaris ×	11	—	19	common	self sterile	H	Valentine, 1947
P. elatior	11				self sterile	H	
Primula elatior ×	11	—	12	rare	self sterile	H	Valentine, 1952
P. veris	11				self sterile	H	
Quercus lyrata ×	—	fertile		rare	———	MM	Yarnell, 1933
Q. virginiana	—				———	MM	
Salix viminalis ×	19	83	—	common	self sterile	MM	Hakanssen, 1929
S. capraea	19				self sterile	MM	
Salvia apiana ×	16	—	2	common	———	N	Epling, 1947
S. mellifera	16				———	N	
Senecio squalidus ×	10	sterile		rare	———	Th.	Rosser, 1955
S. vulgaris	20				self fertile	Th.	Clapham et al., 1957; Fryxell 1957
Silene maritima ×	12	fertile		rare	self fertile	Ch.	Marsden-Jones and
S. vulgaris	12				self fertile	Ch.	Turrill, 1957
Solidago rugosa ×	9	90	68	common	self sterile	H	Goodwin, 1937
S. sempervirens	9				self sterile	H	
Stipa lepida ×	17	sterile		common	———	H	Love, 1946
S. pulchra	32				———	H	
Stipa cernua ×	35	sterile		common	———	H	Love, 1946
S. pulchra	32					H	
Tradescantia subaspera ×	6	partially		common	———	—	Anderson and
T. canaliculata	6, 12	sterile			———	—	Hubricht, 1938

TABLE VII (Cont.)

PERCENTAGE OF FERTILITY, FREQUENCY AND OTHER DATA REGARDING WILD SPECIES HYBRIDS

Species Cross	n	Fertility percentage of F_1 generation		Hybrid frequency in population	Compatibility of parent species	Life form of parent species	Authority
		Pollen	Seed				
Tragopogon dubius ×	6	8	1	rare	———	H	Ownbey, 1950
T. porrifolius	6				———	—	
Vaccinium myrtyllus ×	12	4	—	rare	self fertile	Ch	Ritchie, 1955 (a,b)
V. vitis-idaea	12				self fertile	Ch	
Verbascum nigrum ×	15	sterile		rare	self sterile	H	Focke, 1881
V. thapsus	18				self fertile	H	Clapham et al., 1957
Verbascum nigrum ×	15	sterile		rare	self sterile	H	Focke, 1881
V. phlomoides	16				self sterile	H	Dahlgren, 1948 Fryxell, 1957
Viola riviniana ×	20, 23	sterile		common	self fertile	H	Valentine, 1941
V. reichenbachiana	10				self fertile	H	
Viola rupestris ×	10	sterile		common	self fertile	H	Valentine, 1949
V. riviniana	20, 13				self fertile	H	
Viola arvensis	17	95	—	common	self fertile	Th	Kristofferson, 1923
V. tricolor	13				———	Th	Clapham et al., 1957
Viola lactea ×	29	18	—	common	self fertile	H	Moore, 1958
V. riviniana	20, 23				self fertile	H	
Viola lactea ×	29	fairly fertile		rare	self fertile	H	Moore, 1958
V. canina	20, 23				self fertile	H	Clapham, et al., 1957

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